Effect of Low Moisture Feed Lick Supplementation on Rumen Metabolism in Sheep

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Abstract

The quality and availability of forage varies over the grazing season and at times is nutritionally limiting. Low moisture feed licks (LMFLs) are a commercial supplement retailed as forage balancers that provide additional energy, protein, vitamins and minerals to the diet. Previous research has demonstrated that cattle consuming forage-based diets supplemented with LMFLs have increased voluntary forage intakes, increased digestibility and positive effects on animal performance. The aim of this thesis was to gain a deeper understanding of the biological basis underpinning the effect of LMFL supplementation on metabolism in sheep through a series of *in-vitro*, *in-sacco* and *in-vivo* studies to better explain effects in animal performance.

The effect of LMFL supplementation on the performance of commercial breeding ewes in the uplands was measured. This study confirmed the potential of LMFLs to have a positive effect on ewe live weight and reproductive performance. The effect of LMFL supplementation on rumen function, metabolism and aspects of the rumen microbiome of mature non-productive sheep were measured. LMFL supplementation had no effect on the dry matter degradation of forage *in-vitro* or *in-sacco*, the organic matter, neutral detergent fiber, acid detergent fiber or nitrogen total tract digestibility or voluntary forage intakes. LMFLs had a positive effect on rumen fermentation *in-vitro* with elevated gas production and molar concentrations of the major volatile fatty acids, acetate, butyrate and propionate. However similar observations were not made *in-vivo*. In whole animal trials pre-conditioning the rumen to LMFLs had no effect on the dry matter degradation of forage or rumen fermentation. LMFL supplementation had no effect on the microbial biomass of bacteria, methanogens, or anaerobic fungi recovered from the total solid (SAP) or liquid associated populations. LMFL supplementation and pre-conditioning the rumen to the LMFL had little effect on the structure, diversity or predicted functionality associated with carbohydrate and protein metabolism of the total or potential metabolically active bacterial SAP.

In conclusion, LMFL supplementation has the potential to improve the performance of production animals. However, supplementation does not affect the degradation of forage, voluntary forage intake or rumen microbiota of mature non-productive animals. However, the supplement appeared to be utilised as a substrate for fermentation by rumen microbiota where increases in rumen fermentation products was observed. While the results were limited in these mature animals, the results indicate the potential for greater benefit to be gained from livestock at physiologically demanding times or when actively growing. Hence, further research is required to determine the effect of LMFL supplementation on the metabolism and rumen microbiome of production animals.

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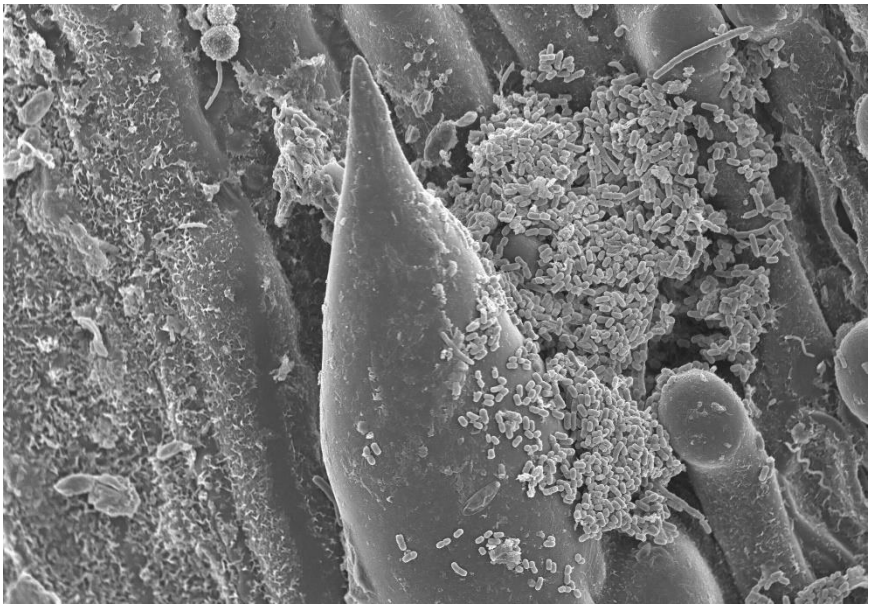
List of Abbreviations

ADF	Acid detergent fiber
ANOVA	Analysis of variance
A:P	Acetate : Propionate
BD	Bottle diet
cDNA	Complementary DNA
CMCase	Carboxymethyl-cellulase
CP	Crude protein
DM	Dry matter
DMD	Dry matter degradation
EE	Ether extract
EAP	Epithelium associated population
EID	Electronic Identification
FME	Fermentable metabolisable energy
ICP-OES	Inductively coupled plasma optical emission spectrometry
LAP	Liquid associated population
LMFL	Low moisture feed lick
LW	Live weight
MANOVA	Multiple analysis of variance
MCP	Microbial crude protein
N	Nitrogen
N-bal	Nitrogen balance
NDF	Neutral detergent fiber
NGS	Next generation sequencing
NPN	Non protein nitrogen
OM	Organic matter
OTU	Operational taxonomic unit
p-value	Probability value
PCA	Principal component analysis
PCoA	Principle component ordination analysis
PCR	Polymerase Chain Reaction
PerMANOVA	Permutational analysis of variance
PGM	Personal genome machine
psi	Pounds per square inch
qPCR	Quantitative polymerase chain reaction
RDP	Rumen degradable protein
RDP	Ribosomal database project
REML	Residual maximum likelihood mixed model
RF	Rumen fluid
rRNA	Ribosomal RNA
RT	Reverse transcription
SABP	Solid associated bacterial population
SAP	Solid associated population
SED	Standard error of difference
SEM	Standard error of mean
VFA	Volatile fatty acids

Chapter 1 Introduction



1.0 Introduction

This thesis aims to investigate the effect of low moisture feed lick (LMFL) supplementation on rumen metabolism and aspects of the rumen microbiome of sheep, to better explain effects on animal performance. This introduction provides background into the global importance of ruminants, their digestive physiology, the rumen microbiome, the degradation and fermentation of dietary substrate by rumen microbiota and the challenges associated within ruminant nutrition.

1.1 Livestock Production

Livestock are an important economic and nutritional commodity for both the developing and developed world. It is estimated that livestock products contribute to around 25% of total human dietary protein consumption and 15 % of human energy consumption (Gerber *et al.*, 2013). Major challenges face the livestock production industry to meet the demand for animal products from the exponential growing human population, which is estimated to reach 11,213 billion by 2100 (UN, 2015, BBSRC, 2017). To accommodate this the FAO (2012) predict that a 70% increase in animal production will be required to meet global demand. There is significant pressure to achieve such demands, sustainably, that is with little competition with humans for land and water for edible food and biofuel production and without compromising capital, animal health and welfare, product quality, retail price and with as little detriment to the environment as possible (Guyader *et al.*, 2016). It is therefore essential to investigate methods of improving the efficiency of livestock production. Nutritional intervention is a fundamental area of research for improving livestock production efficiency, with the ambition of utilising more out of the food fed to animals in the aim of improving animal performance, health and welfare, product quality and reducing margins through reduced on farm variable costs.

1.1.1 Ruminant Products

Ruminants are accountable for around 29% of total global meat production and the majority of global milk consumption (Gerber *et al.*, 2013, Guyader *et al.*, 2016). Ruminants are capable of transforming plant material (lignocellulose) into products (milk and meat) of high nutritional value in the human diet, providing nutrients which may be nutritionally limiting or of limited bioavailability in complete plant-based diets. Dairy products provide a source of protein as well as beneficial fatty acids and micro-nutrients such as, calcium, magnesium, potassium, zinc and phosphorus to human diets (Rozenberg *et al.*, 2016). Red meat (beef, veal and lamb) contains virtually no carbohydrates, is rich in high quality protein (20-24 g/ 100g raw and 27-35 g/ 100g cooked) and contains all eight essential amino acids providing a dietary source of protein for bodily maintenance, growth and repair as well as energy when in surplus (Williamson *et al.*, 2005, Wyness, 2016). Red meat also provides a range of

micro-nutrients such as, vitamins A, B_6, B_{12}, D, E and trace elements iron selenium, zinc, magnesium, cobalt, copper, phosphorus, chromium and nickel which are of importance to human health (Scollan *et al.*, 2006). However, red meat receives negative attention especially from the developed world in relation to consumer perception of negative impacts on health from saturated fat which has been linked with cardiovascular disease and colon cancer (McAfee *et al.*, 2010). The saturated fat (palmitic, stearic, myristic and lauric acid) content of red meat is generally greater in comparison to white meats such as poultry and pork due to the bio-hydrogenation and lipolysis of poly-unsaturated fatty acids within the rumen by rumen microbiota (Shingfield *et al.*, 2013). Poly-unsaturated fatty acids are of great health benefice in the human diet and consist of the essential fatty acids linoleic (n-6) and α-linolenic (n-3) acid which are capable of being transformed in the body into the long chain fatty acids (n-3) EPA and DHA (Scollan *et al.*, 2017). These long chain (n-3) fatty acids are of great health benefit where they have been associated with reducing the incidence of cardiovascular disease, improve ageing (Salter, 2013) and foetal development (Dunstan *et al.*, 2007). In addition, during the biohydrogenation of poly unsaturated fatty acids by rumen microbiota conjugated linoleic acids are produced as intermediary products which have been associated with having anti-obesogenic and anti-atherosclerotic properties (den Hartigh, 2019). Not only do ruminant products provide nutritional benefit to human health, fleece from sheep and hides from cattle and sheep are regularly used for clothing and upholstery (Weimer *et al.*, 2009) thus highlighting the versatility of ruminant products.

1.2 Ruminant Digestive Physiology

Ruminants are land mammals with a unique digestive physiology from dentition (hypsodont) to tract, allowing for successful survival on a completely plant-based diet. It is therefore possible for ruminants to successfully graze marginal land and consume feeds otherwise deemed human inedible such as agro-industrial by-products from the food, drink and bio-fuel industries (Röös *et al.*, 2016).

Mastication of plant material damages plant cell structures, reduces the tensile strength of tissues and increases the surface area of the plant material for increased microbial access within the rumen (Varga & Kolver, 1997, Kingston-Smith & Thomas, 2003). The unique digestive tract of the ruminant consists of a stomach comprised of four compartments (Figure 1.1) termed, the rumen, reticulum, omasum and abomasum (Czerkawski, 1986). The rumen is the largest compartment, is located at the forefront of the digestive tract (McDonald *et al.*, 2010) and is host to diverse consortium of microbiota capable of degrading plant structural carbohydrates into more simple carbohydrates for microbial fermentation, producing products which have utility for both the ruminant host and microbiota.

Digesta of small enough particle size, any unabsorbed by-products of fermentation and microbial biomass are transported from the rumen and filtered through the reticulum and omasum before

entering the abomasum termed the “true stomach”. In the abomasum digesta is subjected to acid digestion before passing into the duodenum of the small intestine for further digestion by mammalian enzymes, digesta then passes to the jejunum and ileum of the small intestine where digestive products are absorbed (Hart *et al.*, 2008). Any unabsorbed or undigested digesta enters the large intestine where secondary fermentation occurs by microbiota in the caecum and colon, any undigested material then passes through the rectum and is excreted as faeces.

Ruminants have the ability to prolong the residency of substrate in the rumen through ruminating which increases the efficiency of digestion (Martin *et al.*, 1999). Rumination is a period of non-food consumption where bi-phasic contraction in the reticulum allows for the regurgitation of substrate from the reticulo-rumen back to the mouth. Regurgitated substrate in the form of a bolus termed “cud” is re-masticated causing further damage to plant cell structures, reducing the particle size of substrate and increasing the surface area of substrate allowing for greater opportunity for microbial access for degradation and fermentation when re-entering the rumen (Watt *et al.*, 2015).

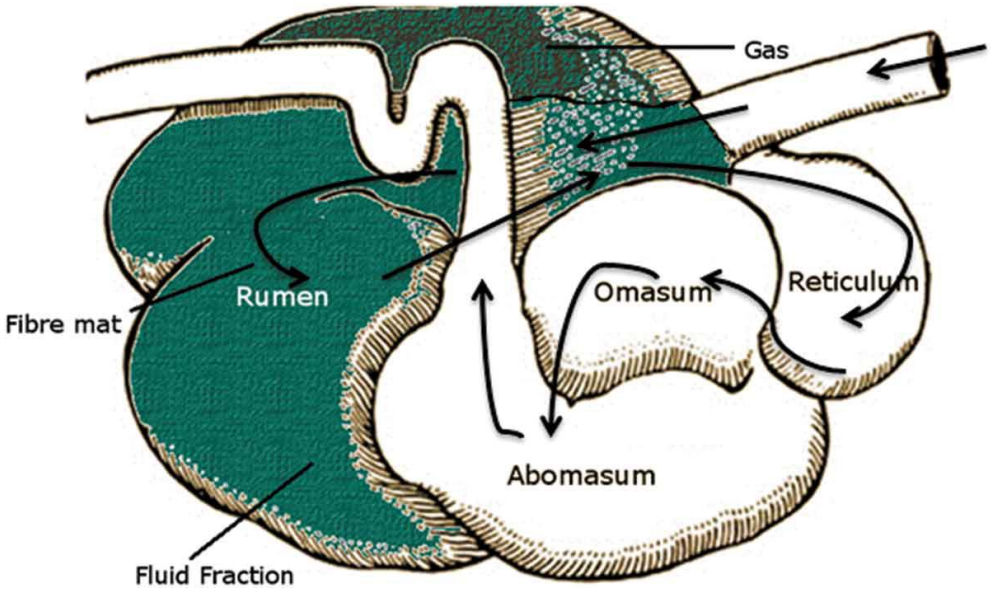


Figure 1.1: Diagram of rumen anatomy highlighting the three states of matter; solid, liquid and gas (Matthews *et al.*, 2019).

1.3 The Rumen Microbiome

The rumen has been described as “the most elegant and highly evolved cellulose-digesting system in nature” (Weimer *et al.*, 2009) and comprises of a large anaerobic chamber with a pH of 5.5–6.5

maintained by phosphate and bicarbonate within saliva (McDonald *et al.*, 2010). The rumen contains three states of matter; solid (dietary substrate), liquid (rumen fluid) and gas (40 % carbon dioxide, 30-40 % methane, 5% hydrogen and trace amounts of oxygen and nitrogen acquired from inhalation during respiration) (Figure 1.1) and is host to a diverse consortium of microbiota of imperative importance to the ruminant host's metabolism (McDonald *et al.*, 2010).

Microbiota resident in the rumen include the kingdoms, bacteria, methanogens, anaerobic fungi, protozoa and bacteriophages whom collectively make up the rumen microbiome (Huws *et al.*, 2018). Microbiome members work in synergy with one another and in symbiosis with the ruminant host (Kumar *et al.*, 2015) to produce and secrete enzymes that the mammalian stomach is incapable of producing itself, allowing for the degradation of complex plant structural carbohydrates (Figure 1.2). This enzymatic intervention allows for the degradation and fermentation of plant material into fermentation products such as, volatile fatty acids (VFAs) providing an essential energy source for the ruminant as well as an energy substrate for microbial growth (Henderson *et al.*, 2015). Volatile fatty acids within the rumen include the short chain fatty acids acetate, propionate and butyrate and branched chain fatty acids valerate, iso-valerate and iso-butyrate derived from amino acids. Acetate, propionate and butyrate are the predominant VFAs totalling 95 % of VFA production (Wanapat *et al.*, 2015) and the diet can significantly influence the proportions of VFAs generated.

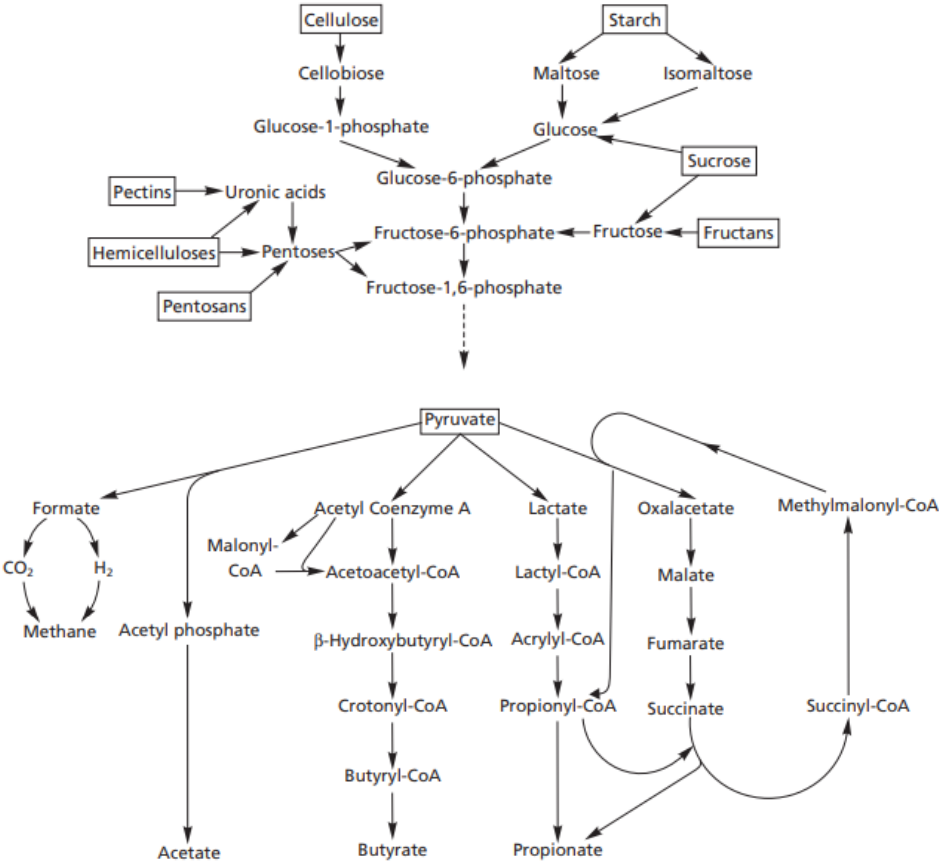


Figure 1.2: Schematic diagram of carbohydrate metabolism in the rumen (McDonald *et al.*, 2010).

Not only are rumen microbiota important in carbohydrate metabolism they also play an important role in protein metabolism (Figure 1.3) where they are responsible for hydrolysing dietary rumen degradable protein (RDP), non-protein nitrogen (NPN) and the branched chain VFAs into amino acids for deamination to ammonia (Kingston-Smith *et al.*, 2008). Provided the fermentable metabolisable energy (FME) content of the diet is sufficient, microorganisms can convert ammonia into microbial crude protein (MCP) (Belanche *et al.*, 2012) which is an important protein source for the ruminant and responsible for 60–85 % of amino acids reaching the small intestine (Hackmann & Firkins, 2015). MCP production within the rumen is a highly inefficient process with as little as 20-30 % of plant protein retained for animal production and 70-80 % excreted in the urine and faeces with detrimental effect to the environment such as contribution to nitrous oxide, greenhouse gas emissions and land and water pollution (Kingston-Smith *et al.*, 2012). Moreover not all MCP reaches the small intestine with up to 50% degraded and recycled in the rumen as NPN (Oldick *et al.*, 2000).

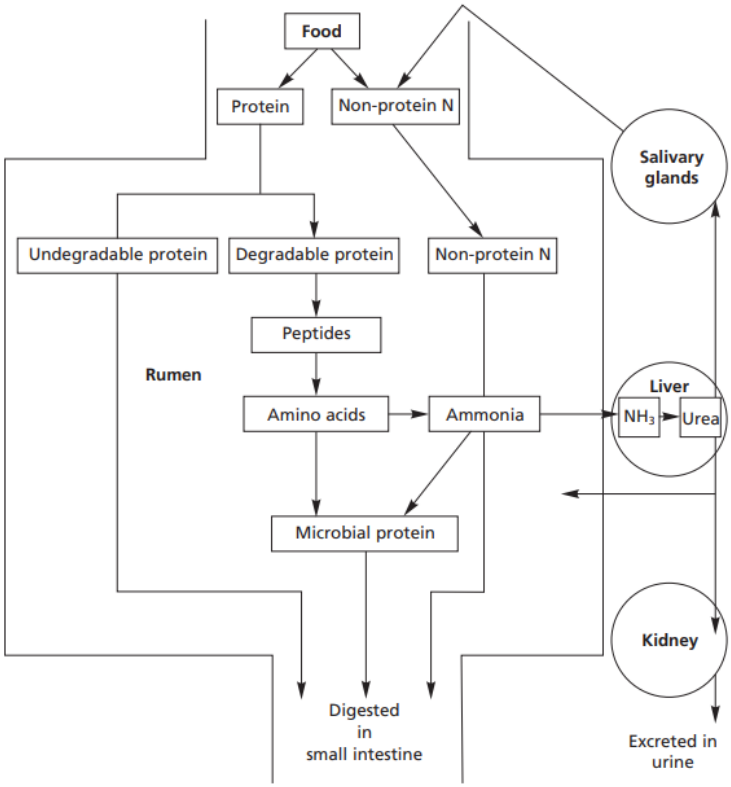


Figure 1.3: Schematic diagram of protein metabolism in the rumen (McDonald *et al.*, 2010).

1.3.1 Rumen Microbiota Sub Populations

The rumen microbiome is comprised of three localised sub-populations, 1) the solid associated population (SAP); 2) the Liquid associated population (LAP); and 3) epithelium associated population (EAP) (Cheng *et al.*, 1980). The most abundant sub-population is the SAP, this community is either firmly or loosely attached to plant material, accounts for up to 75 % of total microbial biomass and is responsible for the enzymatic degradation of complex plant structural carbohydrates into substrates for fermentation (McAllister *et al.*, 1994, Belanche *et al.*, 2017). The LAP accounts for approximately 20-30 % of total microbial biomass and is responsible for the initial colonization of dietary substrate and fermenting soluble and simple carbohydrates (Belanche *et al.*, 2017). The EAP has a commensal relationship with the ruminant host and plays an important role in maintaining the anaerobic environment of the rumen by sequestering oxygen entering the rumen from respiration and the hydrolysis of urea (McAllister *et al.*, 1994).

1.3.2 Rumen Bacteria

Bacteria are present in all three sub populations of microbiota and have been described as the most abundant and diverse kingdom of microorganism within the rumen (Huws *et al.*, 2018). They are thought to account for > 50% of microbial cell biomass (Creevey *et al.*, 2014) and approximately 95% of microbiota within the rumen (Brulc *et al.*, 2009). Bacteria have traditionally been classified into functional groups via their predominant role in pure cultures *in-vitro*, for example fibrolytic (*Ruminococcus albus, Ruminococucus flavefaciens, Fibrobacter Succinogens and Butyrivibrio fibrisolvens*), amylolytic (Selenomonas ruminantium and Streptococcus bovis), proteolytic (*Prevotella spp.*) lipolytic (*Anaerovibrio lipolytica*), lactate producers (*S. bovis and S. ruminantium*) and lactate consumers (*Megasphaera elsdenii*) (Belanche *et al.*, 2012). However research *in-vivo* involving the colonisation of fresh forages suggests microbiota to have different functions to those identified *in-vitro*. Huws *et al.* (2013) identified *Prevotella spp.* to colonise Perennial Ryegrass rapidly suggesting fibrolytic activity. Similar observation was made in an additional study by Huws *et al.* (2016) who also suggested *Prevotella* to have a major role in the degradation of plant material.

Bacteria are able to adhere to dietary substrate attaching themselves to plant cellular structures via adhesion proteins and bacterial glycocalyx (McAllister *et al.*, 1994). Once colonised, bacteria form bio-film complexes, produce a wide variety of enzymes (amylases, cellulases, proteases) and divide producing sister cells enabling plant material to be degraded locally (Varga & Kolver, 1997). Many bacteria have more than one metabolic role within the rumen dependant on rumen conditions. Wirth *et al.* (2018) suggests the existence of a "back up microbial team" with several genera of bacteria having the capability to perform core metabolic functions associated with fiber degradation and fermentation, enabling the successful continuation of metabolism at times where conditions are not optimum.

A "core microbiome" of bacteria is thought to exist in the rumen. Jami & Mizrahi (2012) demonstrated 16 lactating dairy cows fed the same diet had similarities in the abundance and presence of certain bacterial species within rumen fluid. These were primarily *Prevotella*, and *Osciillospira*, *Butyvibrio* and from the family *Lachnospiraceae*. A similar observation was made by Wirth *et al.* (2018) who observed dairy cows in late lactation to all have the presence of certain genera from the phyla *Bacteroidetes*, *Firmicutes*, *Spirochaetes*, *Fibrobacteres*, *Actinobacteria* and *Proteobacteria* with the genera *Prevotella* being the most abundant genera within all rumen fluid samples. This observation has not only been identified locally within a herd but globally. Henderson *et al.* (2015) observed the presence of genera *Prevotella*, *Butyvibrio*, *Ruminococcus*, unclassified genera from families *Lachnopiraceae* and *Ruminococcaceae* and order *Bacteriodales* and *Clostridales* to occur in rumen samples collected

globally from different ruminant species and breeds and from different climates, diets, feeding managements and production systems (Figure 1.4). Wallace *et al.* (2019) also observed a core microbiome to exist when investigating the microbiomes of 1000 dairy cows in 4 European countries. Moreover, Wallace *et al.* (2019) found there to be a link between host genetics and the core microbiota identified in the rumen, suggesting the capability to predict phenotypic traits such as enteric methane emissions, rumen metabolites, blood metabolites and the efficiency of milk production.

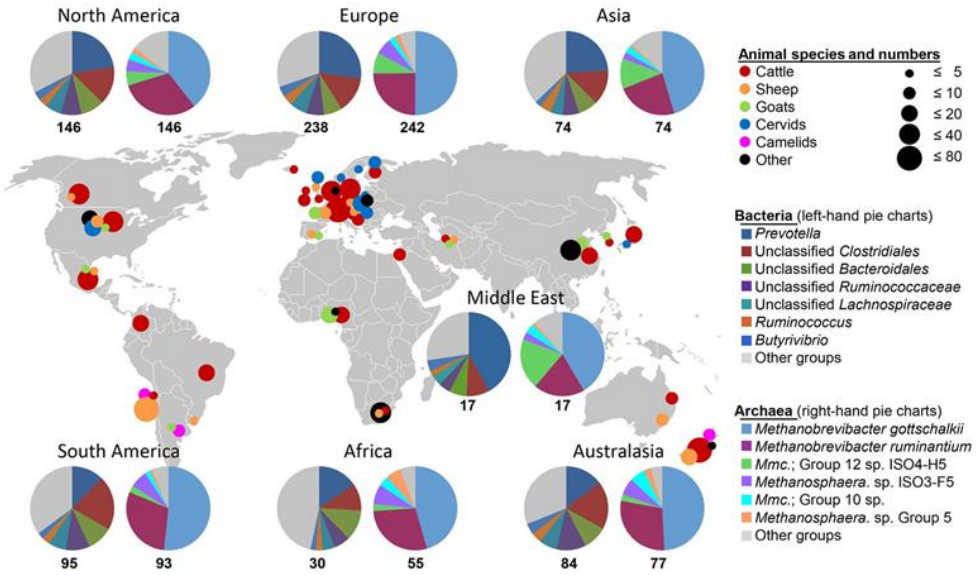


Figure 1.4: Bacteria and Methanogenic communities in different ruminant species and global locations (Henderson *et al.*, 2015).

1.3.3 Rumen Methanogens

Methanogens belong to the kingdom Archaea and have a population density of between 10^8–10^9 ml within rumen fluid (Wang *et al.*, 2017). Methanogens are present in all three sub-populations of microbiota and have methanogenic action in the rumen as part of the end product of their energy metabolism (Greening *et al.*, 2019). They are responsible for transforming the end products of fiber fermentation such as, hydrogen, carbon dioxide, acetate and methylated compounds into methane via reducing carbon dioxide with hydrogen (Evans *et al.*, 2019). Around 80% of methane produced by ruminants is generated in the rumen and expelled into the environment via eructation and the remaining 20% is generated from the decomposition of manure (Vergé *et al.*, 2007). Methane is a greenhouse gas and of great concern regarding its global warming potential which is 28 times greater than carbon dioxide (Breider *et al.*, 2019). Not only is methane environmentally costly, methanogenesis is energetically inefficient with an estimated energy cost of 2-12 % of total gross

energy intake by the ruminant, thereby reducing feed conversion efficiency within the rumen (Johnson & Johnson, 1995).

Although environmentally detrimental and energetically inefficient, methanogenesis is an essential metabolic function in the rumen and without it a build-up of hydrogen would create an environment unfavourable for microbial fermentation (Morgavi *et al.*, 2010). Piao *et al.* (2014) emphasises the importance of the hydrogenotrophic action of methanogens in fiber degradation especially for recalcitrant lignocellulytic material. Therefore it is not surprising that methanogens from the hydrogenotroph family have been identified to exist in the "core microbiome" (Jami & Mizrahi, 2012, Morgavi *et al.*, 2013, Gruninger *et al.*, 2019). In a global rumen microbiota study, Henderson *et al.* (2015) observed *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium* to be the dominant genera of methanogens across a range of ruminant species and breeds within species accounting for 74% of all archaea identified (Figure 1.4). A similar observation made by Wirth *et al.* (2018) whom identified the genus *Methanobrevibacter* to be the predominant genera within the methanogenic community of lactating dairy cows.

1.3.4 Rumen Anaerobic Fungi

Anaerobic fungi of the phylum *Necocallimastigomycota* account for around 20% of rumen microbial biomass (Rezaeian *et al.*, 2004) and are described as one of the most important fiber degrading microorganisms within the rumen (Edwards *et al.*, 2017). Due to their anaerobic nature, these fungi contain hydrogenosomes rather than mitochondria for energy metabolism producing the end products hydrogen, carbon dioxide, formate and acetate (Voncken *et al.*, 2002, Gruninger *et al.*, 2014). As hydrogen and carbon dioxide producers anaerobic fungi have a trophic relationship with methanogens (Li *et al.*, 2019) who are responsible for utilising these gaseous by-products transforming them into methane as described in section 1.3.3. Likewise, a co-culture study using enrichment procedure by (Cheng *et al.*, 2009) demonstrated the ability to achieve a metabolically stable syntrophic community of anaerobic fungi and methanogens in media absent of metabolites favourable for methanogenic culture.

Anaerobic fungi exist in the rumen in two metabolic states, motile free floating in rumen fluid (flagellated zoospore) or vegetative and reproductive adhered to substrate (thallus) (Mountfort, 1987). Zoospores are secreted from the thallus and are responsible for rapidly colonising substrate where they germinate producing penetrative mycelium which damage plant cellular components such as the cuticle on the epidermis (Orpin, 1977). Cellulosomes within mycelium produce and secrete a wide range of enzymes including cellulases, xylanases, mannases, esterases, glucosidases and glucanases which degrade plant structural carbohydrates (Bauchop, 1979, Srinivas & Gupta, 1997,

Gordon & Phillips, 1998). In addition, it is thought anaerobic fungi have a greater capacity for degrading plant fibrous materials in comparison with bacteria (Edwards *et al.*, 2017). The invasive action of mycelium coupled with enzymatic degradation increases the surface area for fermentation of carbohydrates by opportunistic microorganisms (Gruninger *et al.*, 2014). As well as having an active role in fermentation, anaerobic fungi can be an energy source for protozoa. An *in-vitro* study by Miltko *et al.* (2014) demonstrated the capability of ciliate protozoan *Eudiplodinium maggi* to predate on fungal zoospores utilising their cellular carbohydrates such as chitin.

1.3.5 Rumen Protozoa

Protozoa are the largest microorganism (10-200 μm) within the rumen and account for up to 50% of microbial bio-mass in the rumen (Newbold *et al.*, 2015). Protozoa within the rumen can be observed via light microscopy and have been identified via their morphology and classified into the groups ciliate and flagellate. Ciliate protozoa are the most abundant group and are classified into the taxonomic orders *Holotrich* and *Entodiniomorphida* dependant on the location of cilia on their bodies (Dehority, 1993). For example, *Holotrichs* have cilia all over the body and *Entodinomorphida* have cilia in specific regions on the body.

The role of protozoa in the rumen is largely ambiguous. Protozoa are capable of predating on bacteria and other protozoa where they hydrolyse and ferment them into products of utility for the host and other microbiota (Morgavi *et al.*, 2010). Protozoa ingest non-structural carbohydrates such as starches and sugars and thus slow the rate of fermentation down, helping to maintain a high rumen pH (Francisco *et al.*, 2019). *Holotrich* protozoa have the inability to control their sugar consumption and rupture at capacity resulting in a greater number of *Entodinomorphida* present in the rumen when high starch/ sugar diets are fed (Russell, 2002). Protozoa are also large producers of hydrogen which is a substrate for methanogenesis by archaea in which they are exo-symbionts for (Mosoni *et al.*, 2011). Protozoa can have a negative effect on host protein metabolism where they sequester dietary and MCP thus preventing protein sources migrating further down the digestive tract for absorption by the host (Ramos-Morales *et al.*, 2017).

It is possible for ruminants to successfully survive without ruminal protozoa (Newbold *et al.*, 2015), thus research has been conducted into manipulating the rumen to try and reduce protozoal numbers/ completely remove protozoa. Complete removal of protozoa from the rumen (defaunation) has been shown to increase microbial protein supply to the host by up to 30% and reduce enteric methane production by 11% (Ramos-Morales *et al.*, 2017). However, Belanche *et al.* (2011) demonstrated the absence of protozoa in the rumen of lambs from birth had a negative effect on rumen fermentation reflected by reduced forage digestibility and ruminal volatile fatty acid concentrations compared with

faunated lambs. Similar observation was made by Eugene *et al.* (2010) who demonstrated growing lambs to have reduced fiber fermentation in comparison with faunated lambs.

1.3.6 Rumen Bacteriophages

The ruminal bacteriophage (phage) population is estimated at $10^7 – 10^9$ particles per ml (Berg Miller *et al.*, 2012) and is termed the virome (Ross *et al.*, 2013). Phages are capable of infecting and replicating within bacteria and archaea (Klieve *et al.*, 1989, Ross *et al.*, 2013) where they exert lytic (cell rupture) activity resulting in the release of their progeny into the ruminal environment or pseudolysogenic activity where the phage remains dormant in the host cell (Gilbert *et al.*, 2017). Little is known about the presence of different types of phages in the rumen or their direct effect on the rumen microbiome and metabolism (Huws *et al.*, 2018). However, phages are known to influence microbial ecology affecting competition and horizontal gene transfer between bacterial species as well as contributing to the maintenance of bacterial diversity (Berg Miller *et al.*, 2012, Koskella & Brockhurst, 2014).

1.4 Colonisation of Dietary Substrate by Rumen Microbiota

Dietary interactions with rumen microbiota are of great interest to ruminant nutritionists and microbiologists in the aim of improving feed efficiency and animal performance. Rumen microbiota are responsible for the degradation and fermentation of plant material into products of utility to the host such as VFAs and MCP. On entering the rumen substrate is exposed to the rumen microbiome, in which certain communities are attracted to substrate randomly via chemotaxis or adhere to the surface of the substrate and invade through damaged plant tissues degrading plant material from the inside out (Varga & Kolver, 1997).

Initially plants are host to an assortment of colonising endophytic and epiphytic microbiota from their habitual environments (Pontonio, et al. 2018). Exposure of plant material to microbiota within the rumen results in the rapid replacement of the plant endophytic and epiphytic community with rumen microbiota (Huws, et al. 2014). This initial colonisation event is rapid occurring within 5-30 minutes of exposure within the rumen (Edwards *et al.*, 2008, Huws *et al.*, 2013, Koike *et al.*, 2014, Piao *et al.*, 2014, Huws *et al.*, 2016, Cheng *et al.*, 2017, Elliott *et al.*, 2018). The events of colonisation have been widely recognised as biphasic (Huws *et al.*, 2013), with the initial colonisation of substrate undertaken by a primary colonising community before shifting to a secondary colonising community in time (Edwards *et al.*, 2007, Huws *et al.*, 2013, Piao *et al.*, 2014, Liu *et al.*, 2016, Mayorga *et al.*, 2016, Cheng *et al.*, 2017, Elliott *et al.*, 2018). However, Belanche *et al.* (2017) discusses the concept of colonisation

being tri-phasic with the existence of a tertiary colonizing community which he describes as less complicated and slower to establish than the latter communities.

The primary colonising community are the first community to colonise substrate and are responsible for fermenting simple soluble carbohydrates associated with the substrate, participating little or no role in the degradation of insoluble recalcitrant material (Belanche *et al.*, 2017). The primary colonising community is later replaced by a secondary colonising community, which are responsible for producing enzymes for the degradation of insoluble recalcitrant material (Huws *et al.*, 2014, Piao *et al.*, 2014, Liu *et al.*, 2016, Mayorga *et al.*, 2016, Belanche *et al.*, 2017, Cheng *et al.*, 2017, Elliott *et al.*, 2018). This secondary community has been described as more complex and a greater size in comparison with the primary colonising community (Belanche *et al.*, 2017).

1.4.1 Factors Effecting the Microbial Colonisation of Substrate

The degradation and fermentation of substrate within the rumen is largely dictated by the rumen microbiome, the metabolic activity of the microbiome, host metabolism, substrate type and chemical composition (Varga & Kolver, 1997).

The duration of primary colonisation and the shift to secondary colonisation has been demonstrated to vary between plant species, which is thought to be attributed to differences in plant physical and chemical compositions (Huws *et al.*, 2014, Mayorga *et al.*, 2016). The *in-sacco* incubation of fresh Perennial Ryegrass (*Lolium perenne L.*) in non-lactating dairy cows by Huws *et al.* (2013), demonstrated the primary colonising community to establish within 0-2 hours, with a shift to the secondary community post 4 hours of incubation. Such observation was also made with Perennial Ryegrass by Mayorga *et al.* (2016) *in-vitro*. Similar observation was also made by Elliott *et al.* (2018) when investigating the *in-vitro* colonisation of Perennial Ryegrass, however the primary events of colonisation took up to 6 hours before a shift to the secondary colonising community post 6 hours for forages Birdsfoot Trefoil *(Lotus corniculatus*) and Red Clover *(Trofolium pratense*). However, cows in this study had access to grazing and were fed Perennial Ryegrass silage before the study, therefore it is likely that their rumens were more adapted to the Perennial Ryegrass diet.

Forage processing has also been demonstrated to affect the events of colonisation. Using the Rumen Simulation Technique (RUSITEC), Belanche *et al.* (2017) demonstrated the primary microbial colonisation of Perennial Rye grass hay to take twice as long and the shift from the primary to secondary colonising community to take three times as long in comparison with fresh Perennial Rye grass. Likewise, *in-sacco* study using non-lactating dairy cows demonstrated the primary colonisation of conserved forages; Rice Straw (*Oryza sativa L.*), Alfalfa hay (*Medicago sativa*) and Switchgrass

(*Pancium virgatum*) to take up to 6 hours before replacement by the secondary colonising community post 6 hours of incubation in the rumen (Piao *et al.*, 2014, Cheng *et al.*, 2017).

Not only does the chemical and physical composition of plant material have a bearing on the events of microbial colonisation, so does the metabolic activity of plant material. Kingston-Smith *et al.* (2013) suggests that fresh forage on ingestion is still metabolically active and due to physical damage from mastication and exposure to the ruminal environment (temperature, anaerobic, pH), plant cellular stress and defence mechanisms are induced and can result in the self-degradation of certain plant protein and lipid resources, thus effecting the availability of nutrients available. Through the use of metabolite fingerprinting by fourier-transform infrared spectroscopy (FTIR) analysis Kingston-Smith *et al.* (2013) reported variation in the microbial colonisation and fermentation characteristics of different varieties of Perennial Ryegrass of relatively similar chemical composition, suggesting differences to occur in the metabolic response of plant genotypes when exposed to the rumen, thus affecting nutrient availability for microbial growth.

1.5 Ruminant Nutrition in Extensive Grazing Systems

Nutrition is an integral part of livestock production, where nutritional inadequacies such as over and under nutrition can negatively affect animal health and welfare, compromise animal performance and productivity and cause financial detriment to the producer, therefore it is essential that sufficient nutritional management strategies are in place.

Forage is the cheapest and most economical feed resource available for ruminants and is described by the AHDB (2019) as “the most important, yet overlooked, resource for livestock production”. The majority of nutrition for extensive grazing ruminants in the UK is reliant on home grown temperate forages (Kingston-Smith *et al.*, 2013), with the most popular forage being Perennial Ryegrass (*Lolium perenne L.*) due to its fast growing ability in temperate climates (Taweel *et al.*, 2005). However, it is commonly sown with legumes such as and white (*Trifolium repens L.)* and red clover (*Trifolium pratense L.)* to increase yield, protein content and reduce the need for fertiliser application (Grings *et al.*, 2016).

The nutritional quality and availability of forage in temperate climates is highly variable year to year and across the grazing season and at times insufficient to meet animal production demands resulting in negative impacts on animal health, the onset of metabolic disease, poor body condition, performance and productivity (Grings *et al.*, 2016). This is especially true over the winter months during plant dormancy. Bowman *et al.* (1995) describes how plants during periods of dormancy and mature plants have a high dry matter content are low in energy and protein and have reduced

digestibility in comparison to younger plants. Likewise Heitschmidt *et al.* (1995) describes how the leaves of young plants have crude protein contents of 15-20 % at the 2-3 leaf stage and as the plant matures the protein content declines with protein content lowest at 4-7 % when plants are dormant. Similar challenges exist in other climates. For example, in Tibet where the cold season results in a deficit of forage resulting in losses to body condition and poor animal performance (Jing *et al.*, 2018). Likewise in semi-arid, arid and tropical climates during the dry season forage is limiting in availability and nutritional quality due to plant dormancy (Salem & Nefzaoui, 2003). This is particularly problematic for livestock due to limitations in physiological intake capacity and especially for animals at physiologically demanding times such as growth, gestation and lactation and animals of high genetic merit. To compensate conserved forages such as silage and hay are often fed to animals produced from forage when in surplus during the summer months. However the nutritional quality of conserved forages is highly dependent on climate, the plant stage when cutting and the ensiling process and at times can be insufficient to meet the nutritional demands for production (Beever *et al.*, 1986).

Forages can also lack concentrations of minerals sufficient for animal performance. Trace elements such as cobalt, copper, iron, iodine, manganese, selenium and zinc are integral for biological function and thus deficiencies can seriously compromise performance, productivity with disease resulting in cost to animal health as well as economic detriment (López-Alonso, 2012). The mineral content of soil and ground water is largely dictated by rock type (Singh & Schulze, 2015) therefore nutritional deficits are often geographically related as well as resultant of anthropogenic activity. McDowell (1996) describes how plants do not require the trace elements selenium, cobalt or iodine for growth and can grow sufficiently in soils limited in iron, zinc, manganese, copper and cobalt. Likewise the absorption of minerals from the soil and plant roots is highly variable and dependant on extrinsic factors such as soil pH, drainage, moisture content and temperature (Reid & Horvath, 1980). Therefore, supplementation of minerals to the diet may be required, however over supplementation can result in toxicity and economic cost.

To prevent nutritional deficiencies on farm, adaptation of production systems and nutritional management techniques are essential. Such strategies may include, breed selection, timing of physiological events, pre-conditioning animals for weight loss, grass land management, production of additional feeds such as conserved forages and fodder and providing a means of additional or supplementary nutrition (Grings *et al.*, 2016).

1.6 Supplementary nutrition

Supplementary nutrition can be defined as additional nutrition provided alongside the existing diet to satisfy the nutritional maintenance and production requirements of an animal. Supplements provide

one or a combination of additional nutrients to the diet such as, energy, protein, vitamins and minerals. They are either administered directly on an animal to animal basis (trace elements via drench/ bolus) or delivered to the herd/ flock within feed as part of a total or partial mixed ration (feed additive) or placed out at grazing for self-regulatory consumption (roughages, concentrates, liquid feeds, feed blocks and feed licks). It must be remembered that supplementary nutrition is costly, adding to on farm variable costs and for economic viability should only be fed at times when the diet is nutritionally limiting for animal production requirements.

1.6.1 Self-Regulatory Supplementation

Concentrate feeds, feed blocks and feed licks are popular methods of supplementation. These products involve the self-regulatory consumption of the supplement by the ruminant allowing the ruminant to obtain nutrients to appetite. However there are limitations with self-regulatory supplementary feeding such as, difficulties in measuring individual animal intakes, if an animal over/ under consumes, frequency of consumption or if the animal consumes the supplement at all. Taylor *et al.* (2002) demonstrated older ewes within a flock had greater intakes of a feed block supplement in comparison with younger ewes, which was thought to be associated with dominancy and competition within the flock.

Feeding concentrates is a popular and traditional method of supplementation where a certain amount of concentrate is fed to a flock/ herd each day at pasture either as a mix or in pellet or roll form. Concentrates are typically comprised of cereals thus making them costly due to competition with humans for edible food and for use in the bio-fuel industry (FAO, 2008). In addition, there is high labour requirement at feeding, where feeding is required daily and in certain cases twice a day. A cheaper alternative and more convenient method of supplementation are the use of feed blocks and licks which are placed out at pasture in tubs/ buckets for self-regulatory consumption by the ruminant. Not only are feed blocks of nutritional benefit to the ruminant, they can act as carriers for anthelmintics and for sources of NPN (urea) when protein is lacking in the diet (Salem & Nefzaoui, 2003). Likewise, feed blocks and licks can be used as tool for grassland management. Aubel *et al.* (2011) demonstrated the u strategic placing of feed blocks in a rangeland environment to successfully encourage the migration of livestock away from certain grassland areas, suggesting this method could be used to reduce soil compaction, poaching, prevent over grazing and the grazing of certain plant species thereby allowing vegetation to recover as well as improving plant species diversity. However, the location of the supplement may result in localised poaching, compaction and spoilage of the land due to increased animal migration, however this can be resolved with regular rotation of block placement within the field and keeping blocks away from areas of wet land and water sources.

1.6.2 Feed Blocks

Feed blocks are a relatively old supplementary feeding technology with their use first documented in the 1930s as a means of providing additional protein to forage-based diets, in the form of non-protein nitrogen (urea) with salt included in block formulations to prevent overconsumption and the development of urea toxicity (Salem & Nefzaoui, 2003, Makkar *et al.*, 2007). The technology has since evolved with the inclusion of other ingredients into block formulations such agro-industrial by-products for increased energy and protein provision as well as the addition of pre-mixes (minerals and vitamins) to cater for micro-nutrient deficiencies at grazing.

The use of feed blocks are particularly popular in small holdings in semi-arid, arid and tropical climates where blocks are prepared on farm using cold processing techniques. Blocks are prepared by pressing raw ingredients into a mould along with a chemical binder (cement, lime, clay, gypsum, bentonite, linseed powder) and are left to set and air dry (Salem *et al.*, 2000, Salem & Nefzaoui, 2003). Blocks are hydroscopic and therefore have a high moisture content resulting in ease of consumption by ruminants, however this property increases the susceptibility of the block to spoilage by the elements and mould formation which is of economic disadvantage (Salem & Znaidi, 2008).

Cane molasses are the predominant base ingredient in block formulations acting as a carrier for other less palatable ingredients (urea). Moreover, molasses aid the structural integrity of blocks due to its viscous composition. Molasses have a high sugar content (650 g/ kg DM), provide a good source of fermentable metabolisable energy to the diet and are highly palatable (Ewing, 2016), thus helping to encourage block consumption. Aubel *et al.* (2011) demonstrated that cattle grazing in a rangeland environment have a greater affinity to consume minerals when in a molasses block than when based in granular salt alone. The inclusion of molasses based mineral blocks have demonstrated positive effects on animal performance. Azam & Khan (2006) demonstrated zebu cattle consuming straw based diets supplemented with urea-molasses mineral blocks to be of greater live weights and exhibit greater live weight gains in comparison with control animals. Similarly, Rafiq *et al.* (2007) demonstrated ewes supplemented with urea molasses blocks to have greater live weights and conception rates in comparison with control ewes.

The use of locally sourced agro-industrial by-products such as olive cake, citrus pulp, tomato pulp, grape marc and date pulp are popular in block formulations providing an additional means of nutrition at low cost (Salem & Znaidi, 2008). However inclusion is largely dependent on geographical location and seasonal availability and difficulties often arise regarding product storage and inconsistencies in the nutritional composition between batches (Salem & Znaidi, 2008, Molina-Alcaide *et al.*, 2010, Liotta *et al.*, 2019). Salem & Znaidi (2008) demonstrated an olive-cake based feed block to improve lamb

performance as well as reduce the cost of supplementation in comparison to feeding concentrates. Likewise Molina-Alcaide *et al.* (2010) demonstrated the partial replacement of concentrates with an olive-cake based feed block to improve the quality of goat milk. However Yanez Ruiz *et al.* (2004) demonstrated the inclusion of two-stage olive-cake in block formulations to increase the concentration of plant secondary metabolites (condensed tannins) in the diet which had a negative effect on the digestibility of the diet of wethers and goats.

Feed blocks are also a popular method of supplementation in extensive grazing systems in temperate climates such as the UK. However, literature into their effect on metabolism is limited. There are various manufacturing techniques for producing blocks resulting in a range of different products available for purchase commercially. Such blocks include, pressed blocks which involve the compression of raw materials into a block like structure, poured blocks which involve the use of a chemical binder (calcium oxide or magnesium oxide), low moisture feed blocks which are produced from the evaporation of water from dietary ingredients via heating and low moisture feed licks (Crystalyx®, patent GB2426425, US3961081) which involve the extraction of water from raw ingredients via mild heating and vacuuming (personal communication with Crystalyx®-UK).

1.6.3 Low Moisture Feed Licks

Crystalyx® is a commercial low moisture feed lick (LMFL) available in 22.5 and 80 kg tubs, produced and retailed globally by Caltech a division of Carrs Agriculture Ltd (Figure 1.5). These LMFLs are available in a wide range of different products formulated for the different nutritional requirements of sheep, beef, dairy cattle and deer with different products available for different animal physiological statuses and conditions (Caltech-Crystalyx®-UK, 2019). The base ingredient of the LMFLs are cane molasses which attribute to their high palatability and act as a carrier for added ingredients such as, vegetable oil, hipro soya, prairie meal, urea and a pre-mix of vitamins and minerals (Caltech-Crystalyx®-UK, 2019) with specifications dependant on product type.

These LMFLs are described as forage balancers where they are specifically designed to accompany forage, providing additional nutrition to the diet in the form of energy, protein, vitamins and minerals which may otherwise be lacking in forage. Moreover, they are a popular supplementary feed for sheep especially in upland and hill systems where the daily feeding concentrates is not practical and the harsher climates resultant in supplementation being a major requirement. Typical intakes of the LMFLs range from 40-60 g per day for breeding ewes and 200–300 g per day for beef cattle (Caltech-Crystalyx®-UK, 2019).

Figure 1.5: Cross section of a Crystalyx® low moisture feed lick (Caltech-Crystalyx®-UK, 2019).

The manufacture of Crystalyx® LMFLs is unique in that they are produced via a patented (GB2426425, US3961081) cold flow technique which involves the mild heating and vacuuming of molasses to create a semi-solid uniformed dehydrated product (Caltech-Crystalyx®, 2011). The process is unique in comparison with other commercial feed block manufacturing techniques in that the process uses vacuuming aiding the extraction of moisture from raw ingredients to achieve dry matter contents of > 95 %. As a result, LMFLs have a lower moisture content in comparison with other commercial feed blocks attributing to a higher degree of nutrient density. The heat of the saliva and re-absorption of moisture from the environment results in a thin layer of the LMFL dissolving in which the animal consumes via licking (Figure 1.6). However, the block remains solid which prevents overconsumption and encourages the ruminant to consume the lick little and often. The hard structure of the LMFL is also advantageous in that it reduces the incidence of spoilage by the elements. However, this property can be limiting in that it may discourage feeding or result in an insufficient quantity of the LMFL being consumed.

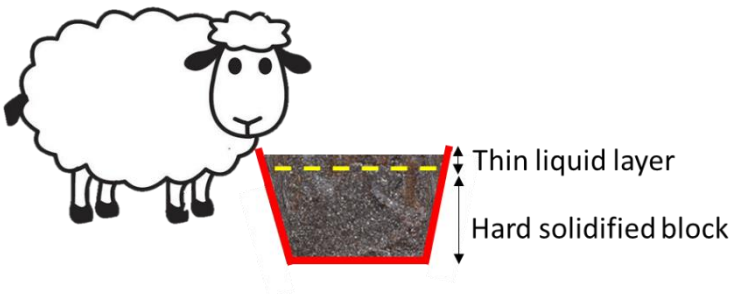
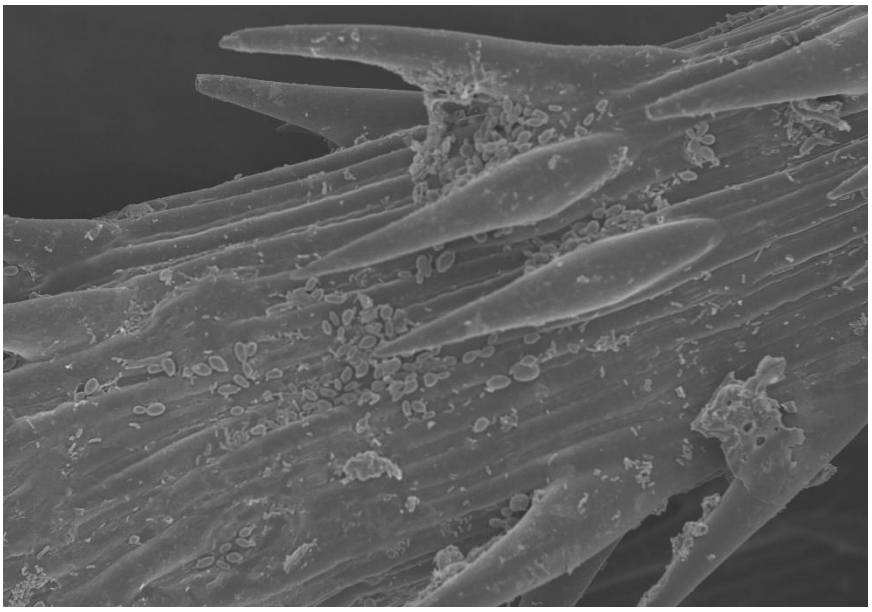


Figure 1.6 Diagram to describe LMFL consumption in extensive grazing systems

1.7 Thesis Aims

The overall aim of this thesis is to gain a better understanding of the biological mechanism by which LMFLs effect the performance of animals. Previous research has demonstrated ruminants supplemented with LMFLs to have positive effects on animal performance in terms of, increased live weight gain and improvements in body condition and blood metabolic status (Cabiddu *et al.*, 2014, Hart & Newbold, 2015). Likewise, the supplementation of poor quality tropical forages with various LMFLs have demonstrated the effect of increasing the digestibility of the diet and in turn have increased voluntary forage intakes (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Leupp *et al.*, 2005, Chaudhry, 2008). It is therefore hypothesised that LMFLs have a complementary effect on poor quality forage-based diets stimulating the metabolic activity of rumen microbiota, thereby increasing the degradation and fermentation potential of forage. However, literature investigating the effect of LMFL supplementation on rumen metabolism and the rumen microbiome is limited, especially in sheep. Through a series of *in-vitro*, *in-sacco* and *in-vivo* experiments, investigations will be made into the effect of LMFL supplementation on the metabolism of sheep, whereby the effect of supplementation on the degradation and fermentation of forage will be investigated in conjunction with aspects of the rumen microbiome. Such information will provide an insight on how LMFLs can be used commercially to improve production efficiency.

Chapter 2 General Materials and Methods



2.0 General Materials and Methods

2.1 Animal Use

All experiments involving the invasive sampling from animals were conducted in compliance with home office regulations, in accordance with The Animal (Scientifics Procedures) Act 1986 (modified by EU Directive 2010/63/EU) and approved by the University's Animal Welfare Ethical Review Board (AWERB) (Aberystwyth_University, 2020). Animals involved were six Aberfield x Texel sheep (D.O.B. 2012, 4 female, 2 male) previously fitted with rumen cannulas (Bar Diamond, Parma Idaho, USA). Sheep were housed indoors in straw pens at the University's farm in Trawsgoed and fed a basal diet of Ryegrass hay *ad-libitum* and approximately 250 g of un-molassed sugar beet pellets (Trident, AB Agri, Peterborough, UK). All sheep had free access to clean fresh water and a salt lick (Baby Pure, Rockies, Winsford, UK) at all times.

2.2 *In-vitro* Gas Production Technique

The *in-vitro* gas production technique is a popular system for screening the degradation and fermentation of feeds and feed additives in a closed system and is relatively non-time consuming and in-expensive to run in comparison with *in-vivo* studies (Carro *et al.*, 2005). The technique involves the incubation of substrate in an inoculum of buffered rumen fluid. The buffer prepared is to the users discretion however, McBee (1953) discusses the benefits of selecting a phosphate and bicarbonate based buffer in *in-vitro* rumen studies, highlighting bicarbonate to be one of the main buffers present in the rumen secreted from the parotid, submaxillary and sublingual glands.

Fermentations typically occur until an asymptote on the gas curve has been achieved. Moreover, following the destructive harvest of bottles there is potential for the measurement of; the quantity of substrate degraded, fermentation parameters and investigations on rumen microbiota (France *et al.*, 2000, López *et al.*, 2007, Cornou *et al.*, 2013, de la Fuente *et al.*, 2017). However, this technique should not be used as an absolute guarantee of what will happen in the rumen as it does not take into account factors associated with animal metabolism such as; organoleptic features of the dietary substrate, feed intake, mastication, salivation, rumination, rumen retention time, absorption of fermentation products and total tract passage rate.

There are many variations of the gas production technique. McBee (1953) describes one of the first designs in which gas was measured via the displacement of fluid using a manometer. Since then methodologies have been simplified. Menke *et al.* (1979) describes an alternative method for measuring gas production where incubations take place in a glass syringe and gas volume measured

via the displacement of the syringe plunger within the barrel. Theodorou *et al.* (1994) describes a further alternative method where gas pressure in the head space of a completely sealed vessel is measured using a portable pressure transducer and the pressure recording used to calculate gas volume according to Boyle's Law as described by López *et al.* (2007). These methods involve the repeated measure of gas readings over time and can be labour intensive and introduce technical error when measuring gas especially between different operators. To overcome this, automated systems such as the Ankom^{RF} Gas Production System (Ankom, New York, USA) have been produced. Here a wireless pressure sensor module with a unique ID chip is attached to bottles which automatically records the pressure difference in the head space of bottles in respect to atmospheric pressure, venting bottles after readings and transmitting data to the operators computer via radio wave (Cornou *et al.*, 2013). However, there is limitation regarding the capacity of sensor modules that can be used in one experiment (n=50).

All *in-vitro* gas production experiments in this thesis were carried out according to the method by Theodorou *et al.* (1994). Experimental designs were based on a previous *in-vitro* colonisation study by Mayorga *et al.* (2016) with modification, where the same ratio of buffer to rumen fluid (1: 9 v/v), substrate dry matter and bottle inoculant were used in respect to vessel volume. Modification of the protocol consisted of bottles being sealed with rubber butyl stoppers allowing access to the head space of the bottle for measuring gas volume as described in section 2.2.4.

2.2.1 Substrate and Supplement

Ryegrass hay was selected as a substrate for all *in-vitro* experiments. Ryegrass hay was selected due to this species of grass being one of the most popular forages grown in the UK for grazing ruminants (Kingston-Smith *et al.*, 2013). Fresh substrate was chopped to 1-2 cm for ease of weighing into bottles and processing residual substrate samples post experimentation. Fresh substrate was used instead of dried substrate as microbial colonisation of forage was to be investigated.

The low moisture feed lick (LMFL), Crystalyx® Extra High Energy (Caltech-Crystalyx®, Silloth, UK) was the LMFL supplement under investigation throughout this thesis. This specific product is one of the most popular products of the Crystalyx® range and is fed commercially in extensive grazing sheep production systems in the UK. This particular LMFL has a high sugar and protein content and is specifically formulated to cater for the production needs of breeding ewes, rams and growing lambs throughout the shepherding calendar. The nutritional specification of the product according to the manufacturer is detailed in Table 2.1. The main ingredients of the LMFL in descending order of inclusion as stated by the manufacturer are; dehydrated cane molasses, pure vegetable oil,

monocalcium phosphate, calcium carbonate, Hipro soya and urea (Caltech-Crystalyx®-UK, 2019). The LMFL did not contain any copper or sodium.

Table 2.1: Nutritional composition of Crystalyx® Extra High Energy according to the manufacturer (Caltech-Crystalyx®-UK, 2019)

Metabolizable energy (MJ/ kg DM)	16.0
Sugar (%)	38.0
Protein (%)	12.0
Protein equivalent of urea (%)	5.00
Fibre (%)	0.25
Ash (%)	17.5
Oil (%)	14.0
Iodine (mg/ kg)	60.0
Cobalt (mg/ kg)	16.0
Selenium (mg/ kg)	9.00
Calcium (%)	3.00
Phosphorus (%)	1.00
Magnesium (%)	0.25
Manganese (mg/ kg)	900
Zinc (mg/ kg)	1800
Vitamin E (mg/ kg)	1000
Vitamin D_3 (iu/ kg)	20000
Vitamin A (iu/ kg)	100000

The supplement was broken via a hammer and chisel into small pieces and weighed into experimental vessels. Attempts were made to transform the LMFL into a fine homogenous powder for ease of weighing however due to the sticky nature and low melting temperature of the product this was not possible. In addition, attempts were also made to freeze dry the LMFL and grind it into a fine homogenous powder, however freeze drying had little effect on the structure of the LMFL most likely

attributed to its unique manufacturing process and as a result of its high dry matter content (> 95%). Attempts were also made to dissolve the LMFL in water/ buffer solution to represent the dissolved state in which it enters the rumen via the saliva. However, pipetting small volumes of the supplement into vessels was time consuming and due to the LMFL containing hipro-soya and vegetable oil resulted in pipette tips getting clogged up and separation of the oil from the sample extracted due to the hydrophyllic nature of the tip.

2.2.2 Rumen Fluid Collection

Rumen fluid was collected from Aberdale x Texel sheep (date of birth 2012; 2 male, 4 female) fitted with rumen cannulas (Bar Diamond, USA) as described in section 2.1. Before morning feeding, rumen digesta was collected through the eye of the cannula and strained through two layers of muslin. The fluid from each sheep was poured into individual pre-warmed thermos flasks with allowance for head space, transported immediately to the laboratory on Penglais campus and stored in an upright incubator (Microbiological Incubator, Jencons-PLS, Leighton Buzzard ,UK) set at 39^{o}C until use. On average the time between rumen fluid collection and experimental setup was approximately 1 - 2 hours.

2.2.3 Inoculum

An inoculum consisting of one part rumen fluid and nine parts phosphate bicarbonate buffer (1:9 v/v) was prepared. One part rumen fluid to nine parts buffer is a relatively weak inoculum in comparison with traditional *in-vitro* gas production studies which typically use 1 : 2 v/v (Menke & Steingass, 1988). One of the aims of the series of *in-vitro* experiments was to investigate the microbiota colonising dietary substrate, therefore it was felt that using a high concentration of rumen fluid would result in a high abundance of microbes and potentially mask the events of colonisation and minor microbes.

The phosphate bicarbonate buffer was prepared the day before the experiment as described by Menke & Steingass (1988) and used to maintain the pH of the rumen fluid in response to likely decline in pH as a result of fermentation of dietary substrate by rumen microbiota. Stock solutions of individual buffer components were prepared as described in Table 2.2 and combined in appropriate proportions to make the buffer solution. The buffer was heated until boiling, removed from the heat and allowed to cool whilst being flushed with a line of CO_2 to create an anaerobic environment. Once cool the buffer was capped and placed in an upright incubator set at 39^{o}C until the morning of the experiment. A reducing agent was freshly prepared on the day of the experiment and added to the buffer as described in section 2.2.4.

2.2.4 *In-vitro* Method

Substrate was weighed into vessels the day before the experiment with 6 mg of substrate being added for every 1 ml of vessel volume. On the morning of the experiment the pre-prepared buffer solution (section 2.2.3) was placed in a water bath set at 39°C and re-flushed with a line of CO_2 to ensure an anaerobic environment. A reducing agent was freshly prepared as described in Table 2.2 and added to the buffer solution. Once the solution had reduced indicated by the solution turning colourless (blue to pink to colourless), rumen fluid was added to create a 1 : 9 v/v inoculum of rumen fluid and buffer. The desired volume of inoculum was dispensed directly into experimental vessels flushed with CO_2 via a Perimatic GP dispensing pump (Jencons Scientific Limited, Leighton Buzzard, UK). Bottles were then stored in an upright standing incubator set at 39°C for the duration of the experiment which was either 24 or 48 hours.

Table 2.2: Individual phosphate bicarbonate buffer components (Menke and Steingass 1988)

Macro mineral solution 1 Litre	
Na_2HPO_4	5.7 g
$KHPO_4$	6.2 g
$MgSO_4 \times 7H_2O$	0.6 g
Micro mineral solution 1 Litre	
$CaCl_2 \times 2H_2O$	132.0 g
$MnCl_2 \times 4H_2O$	100.0 g
$CoCl_2 \times 6H_2O$	10.0 g
$FeCl_2 \times 6H_2O$	8.0 g
Buffer solution 1 Litre	
$NaHCO_3$	35.0 g
$(NH_4)\ HCO_3$	4.0 g
Resazurin solution 1 Litre	
Resazurin	1.0 g
Phosphate buffer composition 1 Litre	
Distilled Water	474.0 ml
Micro Mineral Solution	0.12 ml
Macro Mineral Solution	237.0 ml
Buffer Solution	237.0 ml
Resazurin Solution	1.22 ml
Reducing agent 50 ml / Litre phosphate buffer solution	
1 M NaOH	2.0 ml
$Na_2S \times 9\ H_2O$	313 mg
Distilled Water	47.5 ml

2.2.5 Gas Volume

Gases; hydrogen, carbon dioxide and methane are produced as a by-product of the fermentation of dietary substrate by rumen microbiota (McDonald *et al.*, 2010). Carbon dioxide is also produced as a response of the buffer to a decrease in pH by La Chatiller's principle as a result of volatile fatty acid (VFA) production (section 2.5.2).

The volume of gas present in the headspace of vessels was measured at allotted time intervals. Gas pressure (psi) was measured by piercing the septum of the vessel caps with a hypodermic needle (23 gauge x 1.5 inch) attached to a detachable pressure transducer (Bailey and Mackey Ltd, Birmingham, UK) with digital LED display as described by Theodorou *et al.* (1994) and López *et al.* (2007). Gas pressure was converted into gas volume using equation described by ANKOM (2014).

$$V_x = V_j \times P_{psi} \times k$$

V_x = Gas volume (ml)

V_j = Head space of vessel (ml)

P_{psi} = Pressure in the head space (psi)

k = 0.068004084 (Gas constant-Avogadro's Law of 1 mole gas occupies 25.6 L at 39^{o}C (312 K))

2.2.6 Fermentation Kinetics

Fermentation kinetics were calculated using the exponential equation described by Ørskov & McDonald (1979). Where a+b represents the maximal potential of gas production and c the rate of gas production. This exponential equation can also be used in digestibility studies measuring the extent and rate of degradation of feeds.

$$Y = a + b\,(1 - e^{-ct})$$

Y = Accumulated gas produced over time (ml)

t = Time (Hours)

a = Gas produced at zero hours

b = Gas produced at 24 hours

a+b = Maximal potential of gas production

c = Rate of fermentation

2.2.7 Sacrificial Harvest

At the point of harvest bottles were removed from incubation and placed on ice to inhibit further fermentation. Bottle caps were removed and the solid and liquid fractions separated. Attempts were made to separate fractions by pouring bottle contents through muslin, vacuum filtration using glass crucibles and porcelain Buchner funnels, however the most efficient way of harvest was found to be the pouring of bottle contents through a tea strainer. The residual forage was rinsed with distilled water to remove any loosely attached microbiota and to remove buffer salts. The liquid fraction was mixed to create a homogenous sample and sub-samples recovered for later analysis.

2.3 *In-sacco* Technique

To investigate the degradation of forage within the rumen, the *in-sacco* technique was conducted as described by Huws *et al.* (2016) and Ørskov & McDonald (1979). This technique involved the incubation of pre-weighed nylon bags containing forage in the rumen of sheep for allotted time periods such as; 2, 4, 8, 12, 24 and 48 hours.

All in-sacco experimentations were carried out in six Aberdale x Texel sheep (section 2.1) fitted with rumen cannulas with 3cm lumens (Bar Diamond, Parma Idaho, USA). Fresh forage chopped to 2-4 cm was weighed into pre-weighed 10 x 20 cm nylon bags with 100 μm^2 pore size and secured with elastic bands. Substrate weighed into bags was dependant on the number of bags to be incubated in the rumen at one time. For example, if two bags were to be incubated approximately 8g of dry matter was added to each bag, however if more than two bags were to be incubated approximately 4g of dry forage was added.

Bags were threaded onto 25 cm flexible plastic stalks made in house and capped in place with dimensions based on those described by Preston (1995). Plastic stalks were tied to polyethylene cord attached to the bung of the cannula. Materials, nylon, polyethylene and plastic were specifically selected as they are synthetic and are not able to be degraded by rumen microbiota. Bags were placed one at a time through the eye of the cannula directly into the rumen, due to the eye of the cannula being small a maximum of 4 bags were placed through the cannula and incubated within the rumen at any one time.

2.3.1 Dual Exchange Technique

Where multiple time points for sampling existed (Chapter 5), the dual exchange technique was adopted, due to limitations with the dimensions of the cannula and the physical capacity of the rumen

of sheep. The dual exchange system followed a one bag in one bag out approach resulting in bags being incubated for their allotted time interval, but the initial incubation start time for bags was different.

2.3.2 Harvest

At harvest bags were removed from the rumen and immediately submerged in cold water to inhibit any further fermentation. Bags were rinsed thoroughly by hand for approximately 20 minutes to remove any residual rumen digesta stuck to the outside of the bags and until fluid from bags ran clear on squeezing. Bags were stored at -20^{o}C until frozen and placed onto a freeze drier for 7 days. Post freeze drying bags were immediately placed into a desiccator, re-weighed and the dry matter degradation (DMD) of the substrate calculated as described in section 2.6.2. To account for loss of substrate through the pores of the bags and for loss of soluble fractions of the substrate a control intervention was carried out where bags containing forage were submerged in water and washed for the same allotted time as the *in-sacco* bags. Results were then corrected for losses.

2.4 *In-vivo* Metabolism Study

Six Aberdale x Texel sheep (section 2.1) were used in this study. Sheep were housed in individual metabolic crates, with free access to clean fresh water at all times. Crates were fitted with a filter funnel system designed for the separation of faeces and urine into two separate containers below the crate. The container containing the urine had approximately 100 ml of 10 % sulphuric acid added to maintain the pH of the urine at < pH 3 to prevent the bacterial degradation of purines.

Before morning feeding any feed orts and water left over from the day before were collected. Daily total faeces and urine voided were collected each morning before feeding. The weight of the faeces was recorded, the sample mixed and a representative sub-sample taken and stored at -20^{o}C for analysis. The volume of urine voided was measured and filtered through a layer of muslin to remove debris in the sample such as feed and dust. The pH of the urine was checked and adjusted accordingly (< pH 3) and a representative sub-sample taken and stored at -20^{o}C until analysis.

2.4.1 Diet Delivery

In the appetite study hay was fed *ad libitum* at 2% of each sheep's live weight and fed in two equal proportions am and pm. Initially, forage was to be fed in pelleted form and delivered *ad-libitum* by individual automatic feed dispensers (Nedap, Groenlo, Netherlands) within each pen. Feed dispensers were set up to provide an informative profile of feed intake and behaviour, in which they automatically recorded total daily feed intakes as well as the frequency and duration of feeding. Attached to the

feeders were weigh crates which automatically recorded the live weight of animals every time they entered the crate. Pelleted hay was selected as a feed source due to hay in its natural form not being compatible with the feeder where a hopper dispenser mechanism operated. Pelleted feed would also have increased the reliability of orts measurements in comparison with feeding hay which could easily get mixed in with bedding, stuck to wool or trampled on reducing accuracy of orts recovery. During the pre-adaptation period attempts were made to slowly reduce hay in the diet and increase pelleted hay, however when the diet was nearly all pelleted hay, feed intake reduced and one sheep refused to eat the pellets, therefore it was decided to revert back to feeding hay in its natural form.

LMFLs are hard solidified lick blocks primarily comprised of dehydrated molasses with a DM content of > 95% (Chapter 1, section 1.6.3). The saliva and heat from the saliva melts the lick at point of contact allowing for consumption via licking preventing rapid and over consumption. It was decided to provide a flat rate dose of 70 g/ day due to this thought to be the typical upper limit consumed by sheep grazing extensively. A 5 day pilot study was carried out to determine, if all sheep would consume the LMFL, the daily dose to be offered, roughly how long it took for each sheep to consume the daily dose and the most practical way of supplement delivery. Attempts were made to produce a mini lick by melting the lick into a homogenous layer at a low temperature for consumption via licking, however not all sheep would lick the supplement, finish the entirety of the supplement and tended to grind their teeth on the lick freeing it from the holder or chewed it. There was also concern that re-heating the LMFL may change the chemical and physical composition of the lick and decrease palatability. Heating the lick and leaving the mix to re-set tended to cause the product to become brittle and heating the lick at a high temperature tended to burn the molasses. It was thought that if the standard commercial sized (22.5 kg) lick was made available for each sheep, sheep may overconsume the daily dosage of the LMFL as was experienced by Titgemeyer *et al.* (2004) or even begin to substitute forage for the LMFL due to its high palatability. Therefore the LMFL was delivered to sheep as small chunks as previously delivered to cattle in studies by Greenwood *et al.* (2000), Löest *et al.* (2001) and Leupp *et al.* (2005) and consumption typically occurred within 15-20 minutes of feeding. This method of feed delivery did not match typical consumption by extensive grazing sheep, however it was essential all sheep consumed the daily dose and essential that all of the dose was consumed prior to the rumen study for profiling the effect of LMFL supplementation on the degradation of forage and fermentation in the rumen post consumption.

2.4.2 Total Tract Digestibility

Total tract digestibility of dietary nutrients was calculated using the equation described by McDonald *et al.* (2010).

$$\text{Digestibility of nutrients} = N \times k$$

N = Nutrient in feed (g/kg DM)

k = Digestibility coefficient

$$k = (NC - NF) / NC$$

k = Digestibility Coefficient

NC = Nutrient Consumed (kg/ day)

NF = Nutrient in Faeces (kg/ day)

2.4.3 Nitrogen Balance

Nitrogen balance was calculated using the equation below described by Moorby *et al.* (2002), in which daily nitrogen intake from feed was subtracted from nitrogen present in the faeces and urine. No correction was made for nitrogen losses via the skin or hair, as sheep were not production animals, had only requirement for maintenance, were the same age and experienced both dietary treatments. Therefore it was assumed any nitrogen losses would be the same for each sheep and treatment.

$$\text{Nitrogen Balance} = NI - (NF + NU)$$

NI = Nitrogen intake (g/ day)

NF = Nitrogen in faeces (g/ day)

NU = Nitrogen in urine (g/ day)

2.4.4 Enteric Methane Emissions

Enteric methane emissions were measured from sheep using individual 19.5m^3 indirect open circuit climate control chambers (No Pollution Systems Limited, Edinburgh, UK) with settings at the discretion of the operator. The use of such chambers are recognised as a "gold standard technique" and the most precise method of measuring enteric methane emissions by ruminants *in-vivo* (Garnsworthy *et al.*, 2019). Sheep were housed in in individual metabolism crates situated inside climate-controlled

chambers (Figure 2.1). Chambers were operated at a slight negative pressure with air recirculated around the chamber at by a centrifugal plug fan. Air was extracted from the chamber via an FPZ KO5 extraction blower controlling total air change per hour. Volumetric flow rate of air expelled from the exhaust was calculated using Bernoulli's Principle by measuring the differential pressure along a Venturi pipe every minute via pressure meters (Johnson Controls, Cork, Ireland). Temperature and humidity within the chambers were set to 15^{o}C and 60% humidity respectively (Johnson Controls, UK). Temperature and humidity were measured via sensor probes (Johnson Controls, Cork, Ireland) in the exhaust and the climate adjusted accordingly via an air conditioning unit equipped with chilled water coil and heating elements to maintain an environment of the operator's discretion. A schematic diagram of the chambers can be found in Figure 2.2.

Methane concentration (ppm) was measured as described by Hart *et al.* (2012). Briefly, a continuous sub-sample of air from the exhaust of each chamber was extracted and pumped via plastic tubing directly into the back of ADC MGA-3000 Series Multi-Gas Analyser (ADC Gas Analysis Limited, St Albans, UK) infrared spectroscopy analyser with 8 channel multiplexor previously calibrated with nitrogen gas to zero the analyser and a span gas comprised of a mix of gases of known concentrations. The concentration of methane from each chamber was measured every 10 seconds for 4 minutes simultaneously. The efficiency of the chambers at recovering gas was measured by releasing a known concentration of methane into the chamber at a constant rate via a digital flow meter and applying a correction factor to the data accordingly. The concentration of atmospheric methane was sampled from the air outside of the shed and used to correct the data accordingly. When chamber doors were opened for feeding, cleaning or husbandry purposes, time entering and leaving the chamber was recorded and data removed for that time period.

Daily methane production was calculated using equation:

$$\textbf{Methane (L/ day) = FR x ((Methane x k) / 1000000)}$$

FR = Flow Rate (L/ 24 hours)

Methane (ppm)

k = Correction factor based on chamber efficiency of gas recovery

Figure 2.1: Individual indirect open circuit climate-controlled chambers (No Pollution LTD, Edinburgh, UK)

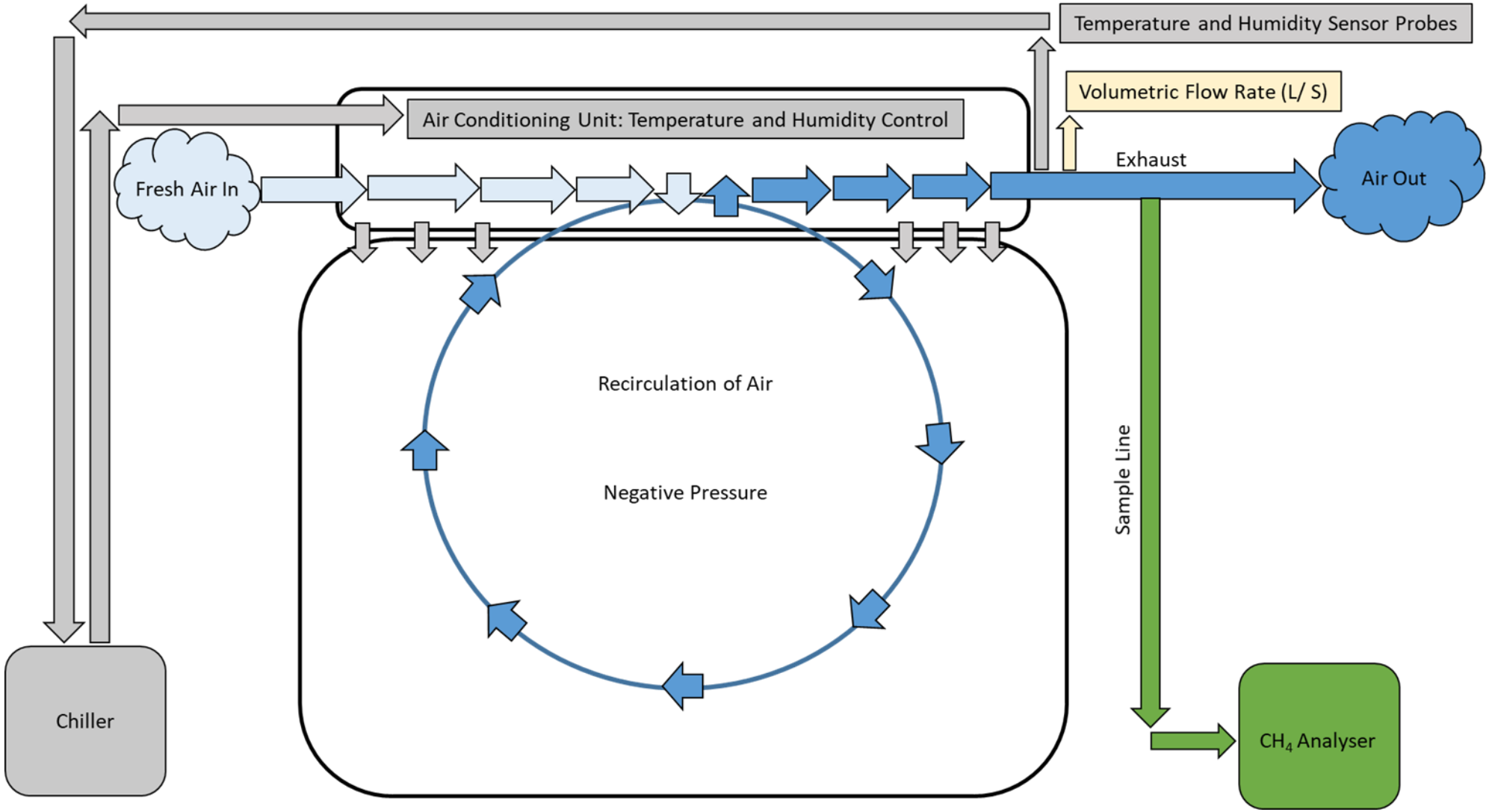


Figure 2.2: Schematic diagram of the climate control chamber operations.

2.5 Analytical Methods: Fermentation Parameters

2.5.1 pH

The pH of inoculum/ rumen fluid was measured by placing the probe of a portable Mettler Toledo SevenEasy pH Meter (Mettler-Toledo, Leicester, UK) into the solution of interest following calibration with solutions of known pH.

2.5.2 Volatile Fatty Acids

Volatile fatty acids (VFAs) are an end product of the fermentation of feeds by microbiota within the rumen (Wang *et al.*, 2016). Molar concentrations of the VFAs, acetate, butyrate, propionate, valerate, iso-valerate and iso-butyrate present in inoculum/ rumen fluid were measured by gas chromatography (GC). Briefly, 1.6 ml of sample was pipetted into 2 ml microfuge tubes containing 400 µl of an internal standard (20% orthophosphoric acid, 20 mM 2-ethylbutyric acid) and stored overnight at 4^{o}C. Samples were centrifuged at 10, 000 xg for 3 minutes at room temperature and the supernatant extracted by syringe and filtered through a 25 mm PES membrane filter with 0.45 µm pore size (Jet Biofiltration, Guangzhou, China) into 2 ml glass GC vials. Vials were capped and crimped and stored at 4^{o}C until analysis.

Vials were placed onto the Varian CP 84000 automatic sampling unit of the Varian CP 3380 Gas Chromatograph (GC) (Varian, Middelburg, Netherlands) for fatty acid analysis which was conducted by Susan Girdwood. One microliter of sample was injected into the GC and detected by flame ionisation. A standard consisting of a mixture of acetic, N-butyric, propionic, valeric, iso-valeric, iso, butyric, N-valeric, iso-caproic heptanonic and formic acids (10 mM Supleco 46975-U) was used to create a calibration curve. Results from the GC analysis were analysed using the Varian Galaxie Chromatography Workstation software version 1.9.3.2. Valerate, iso-valerate and iso-butyrate are branched chain VFAs and have relatively low concentrations within the rumen, therefore the sum of these were calculated and categorised as branched chain VFAs. The ratio of acetate to propionate (A:P) was calculated as an indication of fermentation pathway where acetate is a lipogenic precursor and propionate a glycogenic precursor.

2.5.3 Ammonia

Ammonia is a by-product of fermentation produced when rumen degradable protein, non-protein nitrogen and branched chain VFAs are deaminated by microbiota in the rumen (Kingston-Smith *et al.*, 2008). The molar concentration of ammonia (NH_3-N) within inoculum/ rumen fluid was measured via a phenol hypochlorite assay based on the method by Weatherburn (1967), using reagents alkaline

phenolate, sodium nitroprusside, phosphate buffer and di-sodium EDTA (Table 2.3). A standard curve was prepared using serial dilutions of 20 mg/ l ammonium sulphate (0, 4, 8, 12, 16 and 20 mg/ ml).

Approximately, 800 µl of inoculum/ rumen fluid was added to 2 ml microfuge tubes containing 200 µl of 25 % trichloroacetic acid (TCA) solution. Samples were centrifuged at 10,000 xg for 10 minutes at 4^{o}C. The supernatant was extracted, diluted 1 : 5 v/v with TCA water (80 : 20 distilled water : 25% TCA) and stored at 4^{o}C until analysis. Stock solutions of reagents 1 and 2 (Table 2.3) were freshly prepared on the day of the assay, kept away from direct sunlight and stored at 4^{o}C until use.

On ice, 60 µl of regent 1 was added to the wells of a 98 well microtitre plate (Fisher Scientific, New Hampshire, USA) followed by 10 µl of diluted sample and 100 µl of reagent 2. The plate was immediately placed in a shaking plate Hidex Sense plate reader (Hidex, Osakeyhtiö, Finland), incubated at 39 ^{o}C for 20 minutes before absorbance was read at 660 nm.

Table 2.3: Reagents and stock for phenol hypochlorite assay to determine the concentration of ammonia in samples

Alkaline phenolate stock	
Phenol	11 g
1 M Sodium hydroxide	125 ml
Distilled water	375 ml
Sodium nitroprusside stock	
Sodium nitroprusside	0.05 g
Distilled water	100 ml
Phosphate buffer (pH 12) stock	
Sodium phosphate dibasic	10.65 g
1 M sodium hydroxide	50 ml
Distilled water	950 ml
EDTA stock	
Di-sodium EDTA	4 g
Distilled water	100 ml
Sodium hypochlorite (0.7 % active chlorine) stock	
Sodium hypochlorite	10 ml
Distilled water	90 ml
Reagent 1	
Alkaline phenolate	20 ml
Sodium nitroprusside	100 ml
Di-sodium EDTA (4 %)	5 ml
Reagent 2	
Phosphate buffer	500 ml
Sodium hypochlorite	50 ml

2.6 Analytical Methods: Analytical Chemistry of Feed, Digesta and Excreta

2.6.1 Dry Matter

The dry matter (DM) content of samples was determined by weighing a known weight of fresh sample into a pre-weighed receptacle. Samples were either freeze dried or oven dried at 60^{o}C for 48 -72 hours. Post drying samples were immediately placed in a desiccator and re-weighed when cool. The DM content was calculated using the equation below.

$$\textbf{Dry matter (\%)} = 100 \times (W_3 - (W_1 \times C_1)) / W_2$$

W_1 = Receptacle weight (g)

W_2 = Original sample weight (g)

W_3 = Sample + receptacle weight post drying process (g)

C_1 = Sample blanks (receptacle) (g)

2.6.2 Dry Matter Degradation of Substrate

The dry matter of the substrate at the start of the experiment and the residual substrate at the end of the experiment was determined as described in section 2.6.1. The percentage of dry matter that had degraded was calculated using the equation:

$$\textbf{Dry matter degradation (\%)} = ((DM_B - DM_A)/ DM_B) \times 100$$

DM_B = Dry matter start (g)

DM_A = Dry matter end (g)

2.6.3 Milling

Samples were milled to create a homogenous powder for analysis. Samples were either freeze dried or oven dried at 60^{o}C for 48-72 hours to remove water from samples. Post drying samples were immediately placed in a desiccator and ground using a ball mill (IKA, Staufen, Germany) fitted with a 2 mm dye.

2.6.4 Neutral Detergent Fibre

Neutral detergent fibre (NDF) is a measure of the proportion of insoluble fibre in organic matter which consists of cellulose, hemicellulose, lignin and silica (Van Soest *et al.*, 1991). NDF was measured using method 13 by Ankom Technology ANKOM (2017).

Approximately 0.5 g of ground dried material was weighed into pre-weighed Ankom F57 filter bags and heat sealed. Bags were placed into an Ankom 2000 Fibre Analyser (Ankom Technologies, New York, USA) along with approximately 2 L of NDF solution (Table 2.4) and agitated at 100°C for 75 minutes. All samples were run in triplicate. Empty fibre bags were included in each run for the correction of any loss of bag weight during the NDF procedure.

Table 2.4 : Neutral detergent fibre solution pH 6.9-7.1 (ANKOM 2017)

Chemical	Quantity
Sodium dodecyl sulphate	30.00 g
Di sodium EDTA (dehydrate)	18.61 g
Sodium borate	6.81 g
Sodium phosphate dibasic (anhydrous)	4.56 g
Triethylene glycol	10.00 ml
Distilled water	Made up to 1 litre

Bags were rinsed with warm water to remove residual NDF solution, blotted and submerged in acetone for 3 minutes to remove moisture. Bags were air dried for an hour to allow for the evaporation of acetone and placed in an oven at 100°C for 4 hours. Samples were removed from the oven, placed in a desiccator to cool and re-weighed.

NDF was calculated using the equation:

$$\text{Neutral detergent fibre (\%)} = 100 \times (W_3 - (W_1 \times C_1)) / W_2$$

W_1 = Original bag weight (g)

W_2 = Original sample weight (g)

W_3 = Sample + bag weight post NDF extraction process (dried) (g)

C_1 = Sample blanks (filter bag) (g)

2.6.5 Acid Detergent Fibre

Acid detergent fibre (ADF) is a measure of the proportion of cellulose, lignin and silica in organic matter (Van Soest *et al.*, 1991) and was measured using method 12 by Ankom Technology (ANKOM, 2017). Bags retained from the NDF procedure were placed into the Ankom 2000 Fibre analyser (Ankom

Technologies, New York, USA) with approximately 2 L of ADF solution (Table 2.5) and heated and agitated at 100 °C for 60 minutes. Bags were dried and processed for weighing as described in section 2.6.4.

Table 2.5: Acid detergent fibre solution (ANKOM 2017)

Chemical	Quantity
CTAB	20 g
1 N Sulfuric acid	1000 ml

ADF was calculated using the equation:

$$\text{Acid detergent fibre (\%)} = 100 \times (W_3 - (W_1 \times C_1)) / W_2$$

W_1 = Original bag weight (g)

W_2 = Original sample weight (g)

W_3 = Sample + bag weight post ADF extraction process (dried) (g)

C_1 = Sample blanks (Ankom filter bag) (g)

2.6.6 Nitrogen and Crude Protein

Approximately 200 mg of ground dried sample/ 200 µl urine was weighed into metal crucibles and loaded onto a Variomax Cube Elemental Analyser (Elementar, Langenselbold, Germany). The percentage of nitrogen (N) in samples was measured and converted into the percentage of crude protein in the sample using the equation:

$$\text{Crude protein (\%)} = \text{\% nitrogen in sample} \times 6.25$$

2.6.7 Ether Extract

Measurement of the ether extract (EE) was carried out to determine the fat content in samples using AOCS Official Procedure Am 5-04 by Ankom Technology (ANKOM, 2001).

Approximately 0.5 g of dried ground material was weighed into pre-weighed Ankom XT4 filter bags (Ankom Technology, New York, USA) and heat sealed. All samples were run in triplicate. Empty filter

bags were included in each run for the correction of any loss of bag weight during the extraction procedure.

Bags were placed into an Ankom XT15 Crude Fat Analyser (Ankom Technologies, New York, USA.) and fat was extracted from samples using petroleum ether heated to 60°C for 90 minutes. Bags were removed from the analyser, air dried for 30 minutes and placed in a 100°C oven for 2 hours to dry. Post drying samples were immediately placed in a desiccator to cool and re-weighed.

Ether extract was calculated using the equation:

$$\textbf{Ether extract (\%)} = 100 \times (W_3 - (W_1 \times C_1)) / W_2$$

W_1 = Original bag weight (g)

W_2 = Original sample weight (g)

W_3 = Sample + bag weight post EE extraction process (dried) (g)

C_1 = Sample blanks (filter bag) (g)

2.6.8 Organic Matter and Ash

The organic matter (OM) and ash content of samples was measured by completely burning all organic material in the sample leaving ash residue.

Approximately 0.5 g of dried ground sample was weighed into pre-weighed thermostable crucibles and placed into a muffle furnace to completely ash at 500°C for 24 hours. All samples were run in triplicate. Empty crucibles were run as blanks to account for any moisture within the crucibles that may result in weight loss during the procedure. Crucibles were removed from the furnace, placed in a desiccator to cool and re-weighed.

Ash was calculated using the equation:

$$\textbf{Ash (\%)} = 100 \times (W_3 - (W_1 \times C_1)) / W_2)$$

W_1 = Original crucible weight (g)

W_2 = Original sample weight (g)

W_3 = Sample + crucible weight post ash process (dried) (g)

C_1 = Sample blanks (crucible) (g)

Organic matter was calculated using the equation:

Organic matter (%) = 100 - % ash (%)

2.7 Enzyme Activity of Rumen Microbiota

Enzymatic analysis was conducted as an indicator of microbial activity. The enzymatic activity of amylase, carboxymethyl-cellulase (CMCase) and xylanase produced by rumen microbes was measured via a series of reducing sugar assays as described by (Colombatto & Beauchemin, 2003, Giraldo *et al.*, 2008, Belanche *et al.*, 2016) with modification as described below.

2.7.1 Enzyme Extractions

Freeze dried material was ground to a homogenous powder using liquid-N and approximately 50 mg weighed into 2 ml micro centrifuge tubes (Sarstedt, Nümbrecht, Germany) along with 200 mg of 1 mm fine glass beads (Fisher Scientific, New Hampshire, USA). To tubes, 1.6 ml of cold 0.1 M potassium phosphate buffer (pH 6.8) was added and samples were bead beaten in a Mini-Beadbeater 96 for 3 minutes (BioSpec Products, Oklahoma, USA). Samples were centrifuged at 5000 rpm for 10 minutes at 4^{o}C and the supernatant extracted and stored at -80^{o}C until further analysis.

2.7.2 Substrates

Enzymatic activities of amylase, CMCase and Xylanase were measured using substrates; starch-soluble, carboxymethyl-cellulose and xylan from beechwood (Sigma-Aldrich, Missouri, USA) respectively. Substrates were prepared by dissolving 1 g of substrate in 100 ml of 0.1 M potassium phosphate buffer (pH 6.8).

2.7.3 Reducing Sugar Assay

Assays were conducted in 20 ml glass tubes with relevant substrate and sample blank controls as described in Table 2.6. Blanks were used to correct for any autolysis of substrate/ sample during incubation and for any reducing sugars present in the sample. Samples and control blanks were all run in triplicate. A standard curve was generated for each assay involving serial dilutions of 2 mg/ l glucose at concentrations 0, 0.4, 0.8, 1.2, 1.6 and 2 mg/ ml for the amylase and CMCase assay and serial dilutions of 1 mg/ ml at concentrations 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/ ml of xylose for xylanase assay.

To tubes, buffer and substrate were added where appropriate (Table 2.6) and incubated in a water bath at 39 ^{o}C for 15 minutes. After incubation, 100 µl of sample was added to tubes where appropriate and tubes incubated for at 39 ^{o}C for a further 15 minutes. Tubes were removed from incubation and placed in ice cold water to inhibit any further enzymatic activity. To tubes, 1.5 ml of di-nitro salicyclic

acid (DNS; 10g sodium hydroxide, 10g 3,5 di-nitro salicylic acid, 2 g phenol crystals 99%, 400 g potassium sodium tartrate, 0.5 g sodium sulphite anhydrous / litre distilled water) was added and tubes were placed in a boiling hot water bath for 5 minutes to allow the DNS solution to bind to any reducing sugars present in tubes. Tubes were removed from the water bath and placed in an ice cold water bath to cool. Once cool tubes were placed in a 6715 UV/ Vis spectrophotometer (Jenway, Stone, UK) and absorbance read at 540 nm.

Table 2.6: Reducing Sugar assay experimental setup

Measure	Buffer (µl)	Substrate (µl)	Sample (µl)	Total Volume (µl)
Substrate blank	750	250	0	1000
Sample blank	900	0	100	1000
Sample	650	250	100	1000

2.7.4 Bradford Assay

To determine enzymatic activity relative to the quantity of protein within samples the Bradford assay was conducted using the 96 well plate assay protocol by Sigma-Aldrich, (2018).

Samples were processed as described in section 2.7.1 and all samples were run in triplicate. A standard curve was prepared using serial dilutions of bovine serum albumin (BSA) at concentrations 0, 0.2, 0.4, 0.8 and 1.2 mg/ ml.

At room temperature 5 µl of enzyme extract and 250 µl of Bradford reagent were added to the wells of a 98 well microtitre plate (Fisher Scientific, New Hampshire, USA). Plates were shaken at room temperature on a VWR® Microplate Shaker (VWR International, Pennsylvania, USA) for approximately 30 seconds before being left to stand for 40 minutes at room temperature. Plates were placed in a Hidex Sense plate reader Spectrophotometer (Hidex, Osakeyhtiö, Finland) and absorbance measured at 595 nm.

2.8 Identification of Rumen Protozoa

Protozoa were identified by their morphology via light microscopy. Rumen fluid was collected from sheep (section 2.1) fitted with rumen cannulas as described in section 2.2.2. To 2 ml microfuge tubes 500 µl of strained rumen fluid was added along with 500 µl of saline formalin solution. Tubes were inverted to mix contents and stored in a dark cupboard at room temperature until analysis.

Protozoa were identified via light microscopy using method described by Dehority (1984) and de la Fuente *et al.* (2017). Approximately 24 hours before sample analysis two drops of methylene blue dye was added to samples and incubated at room temperature. On the day of analysis 20 µl of sample was pipetted onto a glass microscope slide and covered with a cover slip. Protozoa identified were categorised into the following taxonomic orders *Holotrichs* and *Entodiniomorphida* (Figure 2.3) as described by Dehority (1993). Any unidentifiable protozoa were categorised into the category "other" to allow for calculation of total protozoa count per 20 µl of rumen fluid sample. All protozoa analysis was carried out by Daiki Matsumoto a student visiting the University as part of his Erasmus programme.

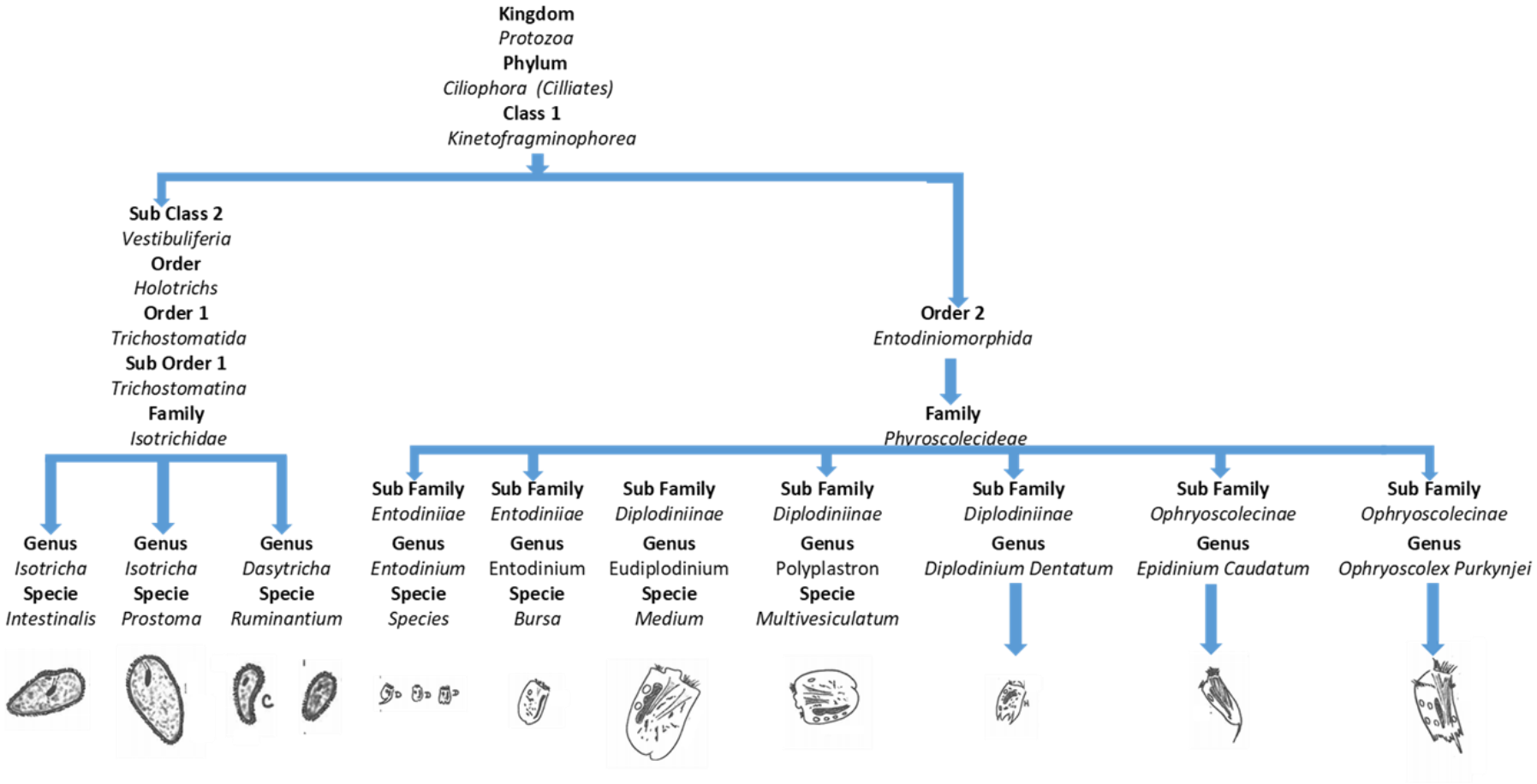


Figure 2.3: Taxonomic classification of rumen cilliate protozoa adapted from Dehority, et al. (1993)

2.9 Molecular Techniques: Quantification of Microbial Biomass

The abundance of microbial biomass was quantified in relation to an environmental sample as an indication of potential microbial growth and activity. Nucleic acids DNA and RNA were extracted in representation of the total and potential metabolically active microbial population respectively. Nucleic acid extraction involves three components, 1) cell lysis; 2) removal of organic and in-organic material that are not associated with nucleic acids; 3) purification of extracts to prevent the degradation of nucleic acid material (Lever *et al.*, 2015). The relative abundance of microbial biomass was quantified via quantitative polymerase chain reaction (qPCR) using specific primers to target different kingdoms of microbiota.

2.9.1 DNA Extraction

DNA was extracted from samples using the extraction method by Yu & Morrison (2004) with adaptation by Minas *et al.* (2011).

To 2 ml micro-centrifuge tubes (Sarstedt, Nümbrecht, Germany), 25-30 mg of freeze dried sample, 2 tungsten beads and 600 µl of lysis buffer (Table 2.7) were added. Tubes were capped and subjected to bead beating in a Mini-Beadbeater 96 (BioSpec Products, Oklahoma, USA) for 30 seconds. Tubes were then incubated at 95^{o}C for 10 minutes before being centrifuged at 5000 xg for 1 minute at room temperature. To tubes, 60 µl of 3M potassium acetate was added. Tubes were mixed via inversion and incubated on ice for 5 minutes.

Table 2.7: Lysis buffer for DNA extraction Minas, et al. (2011)

Chemical	Quantity
500 mM EDTA pH 8	10 ml
1 M tris hydrochloric acid pH 8	5 ml
5 M sodium chloride	10 ml
Ultra-pure water	Made up to 100 ml
SDS	4 g

Post incubation on ice, samples were centrifuged at 17,000 xg at room temperature for 5 minutes and approximately 400 µl of supernatant was extracted and placed into clean 1.5 ml microfuge tubes. To tubes, 50 µl of 5 M sodium chloride and 50 µl of cetrimonium bromide / sodium chloride was added,

tubes were vortexed, incubated at 60°C for 5 minutes, re-vortexed and incubated again for a further 5 minutes at 60°C.

Post incubation, 300 µl of chloroform iso-amyl alcohol (24:1 v/v) was added to tubes, tubes were mixed via inversion to create an emulsion and centrifuged at 17, 000 xg at room temperature for 5 minutes. The aqueous layer of the sample was transferred into a clean 1.5 ml microfuge tubes along with 300 µl of Isopropanol. Tubes were mixed via inversion and centrifuged at 17,000 xg at room temperature for 1 minute.

The supernatant within the tube was discarded leaving a pellet at the bottom of the tube. The pellet was rehydrated for washing in 500 µl of 70 % ethanol and tubes were centrifuged at 17,000 xg at room temperature for 1 minute. The supernatant was discarded, and the ethanol wash repeated.

Pellets within tubes were air dried at room temperature for approximately 30 minutes before being re-suspended in 100 µl of molecular grade water. Tubes were incubated on ice for 30 minutes and DNA quantified as described in section 2.9.3. Samples were stored at -20°C until analysis.

2.9.2 RNA Extraction

RNA was extracted from samples using extraction method described by by Ougham & Davies (1990). Approximately 0.25 – 0.5 g of fresh frozen plant material was placed in a pestle and mortar and ground into a fine powder under liquid nitrogen. To the ground sample, 2 ml of aqua-phenol and 2 ml of warm buffer solution (Table 2.8) was added, contents were mixed and transferred into a sterile 15 ml centrifuge tube (Sigma-Aldrich, Missouri, USA) for incubation at 65°C for 30 minutes.

Table 2.8 Lysis buffer for RNA extraction Ougham and Davies, (1990).

Chemical	Quantity
200 mM sodium acetate pH 5.2	6.7 ml
10 mM di-sodium EDTA	2.0 ml
1 % w/v sodium dodecyl sulphate	10.0 ml
Ultra-pure water	81.3 ml

Post incubation, samples were vortexed, and 2 ml of chloroform added. Tubes were mixed via inversion to create an emulsion, re-vortexed and centrifuged at 20,000 xg at room temperature for 30

minutes. The lower layer of the sample was discarded, and 2 ml of chloroform added, vortexed and centrifuged at 20, 000 xg at room temperature for 20 minutes. The aqueous phase of the sample was removed, transferred into a sterile 15 ml centrifuge tube (Sigma-Aldrich, Missouri, USA) along with 2 ml of chloroform. Tubes were vortexed and centrifuged at 20,000 xg at room temperature for 20 minutes.

The aqueous phase was removed, volume recorded and transferred into 2 ml micro centrifuge tubes (Sarstedt, Nümbrecht, Germany). One quarter of the sample volume of 10 M lithium chloride was added to tubes to achieve a final volume of 2 M, tubes were vortexed and incubated at 4^{o}C for 12 hours to allow the precipitation of RNA out of solution.

Tubes were centrifuged at 20,000 xg at 4^{o}C for 30 minutes to reveal a pellet and the supernatant discarded. Pellets were re-suspended in 1 ml of ice cold 2 M lithium chloride, vortexed and centrifuged at 20,000 xg at 4^{o}C for 20 minutes. The supernatant was discarded and the 2M lithium chloride wash repeated.

Pellets were re-hydrated in 1 ml of ice cold 80 % ethanol, vortexed and centrifuged at 20,000 xg at 4^{o}C for 20 minutes. The supernatant was discarded, and the ethanol wash repeated. Pellets were left to air dry at room temperature for approximately 30 minutes before re-suspension in 20 µl of molecular grade water. Samples were incubated on ice for approximately 20 minutes before quantification as described in section 2.9.3. Samples were stored at -80^{o}C until analysis.

2.9.3 Quantification of Nucleic Acids by Spectrophotometry

The concentration of nucleic acids in extracts were quantified and quality checked using a spectrophotometer. One µl of sample was pipetted onto either a Nanodrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, New Hampshire, USA) or Epoc Micro-Volume Spectrophotometer (BioTek, Winooski, USA) and absorbance read at 260/ 280 nm. Samples were discarded and extractions repeated for samples whose ratio at 260/ 280 nm was not between 1.8 and 2. Such samples were discarded due to extracts being of a poor quality.

2.9.4 Reverse Transcription of RNA into DNA

RNA is relatively unstable and rapidly degraded whereas DNA is fairly robust and easier to handle (Tan & Yiap, 2009), therefore RNA was transformed into complementary DNA (cDNA) via reverse transcription.

RNA was extracted from samples as described in section 2.9.2 and quantified as described in section 2.9.3. Samples were reverse transcribed into complementary cDNA using qPCRBIO cDNA Synthesis kit

(PCRBiosystems, London, UK) which involved the use of random hexamers. A master mix was prepared of 4 µl buffer and 1 µl of reverse transcriptase per sample and 5 µl was added to the wells of a 98 well plate (Thermo Fisher Scientific, New Hampshire, USA). The concentration of RNA in samples was normalised to 50 ng/ µl and the reaction mixture made up to a final volume of 20 µl with molecular grade water. The plate was covered with plastic film and loaded into a T100^{TM} Thermal Cycler (BioRad, California, USA) and run at the manufacturers recommended settings of 42^{o}C for 30 minutes, 85^{o}C for 10 minutes and cooling infinitely at 4^{o}C. Samples were stored at -20^{o}C until analysis.

2.9.5 Quantitative Polymerase Chain Reaction (q-PCR)

Using the quantitative polymerase chain technique (q-PCR), the abundance of microbial biomass from different kingdoms (total bacteria, methanogens, anaerobic fungi and protozoa) were quantified relative to an environmental sample consisting of a serial dilution of standards (10^{-1} – 10^{-5}) previously created from the rumen fluid of cows as described by Belanche *et al.* (2012). Briefly, standards were prepared in-house and involved the extraction of DNA from cow rumen fluid as described in section 2.9.1, amplification of the target gene for the kingdom of interest using specific primers (Table 2.8) via the polymerase chain reaction (PCR) technique, and quantification of the PCR product as described in section 2.9.3. A set of standards of the PCR product were generated by making serial dilutions which underwent qPCR to determine the ct value and estimated copy number in relation to the quantity of DNA within the standard and the estimated length of amplicon. A standard curve was generated and used to calculate the relative abundance of the different kingdoms of microbiota in relation to the estimated copy number.

DNA and RNA were extracted from samples as described in section 2.9.1 and 2.9.2 respectively and quantified as described in section 2.9.3 and RNA was reverse transcribed into cDNA as described in section 2.9.4. Nucleic acids were diluted 1 : 10 v/v with molecular grade water and 1 µl of sample was pipetted into the well of a 384 well plate. All samples were run in triplicate. A master mix was made using 2x PCRBIO SyGrenn Mix Lo-Rox kit (PCRBiosystems, London, UK) with specific primers (Table 2.9) at a concentration of 50 µM. Breifly, 6.25 µl SyGreen Mix, 0.1 µl reverse primer, 0.1 µl forward primer, and 5.05 µl PCR grade water per sample was made and 11.5 µl pipetted on top of the sample to give a reaction volume of 12.5 µl.

The plate was covered with a plastic sealing foil (Roche, Basel, Switzerland), centrifuged to mix contents and placed into a LightCycler® 480 machine (Roche, Basel, Switzerland). The plate was incubated for 95^{o}C for 5 minutes followed by 60 cycles of 95^{o}C for 15 seconds, primer specific melting

temperature (Table 2.9) for 30 seconds, 72^{o}C for 30 seconds and 95 ^{o}C 15 seconds. Melting curve analysis was performed at the end of the cycle to determine primer specificity.

Table 2.9: Forward and reverse primers for total bacteria, methanogens, anaerobic fungi and protozoa for quantitative polymerase chain reaction

Kingdom	Forward Primer	Reverse Primer	Annealing Temperature (°C)	Melting Temperature (°C)	Size (base pairs)
Total Bacteria[a]	GTGSTGCAYGGYTGTCGTCA	ACGTCRTCCMCACCTTCCTC	61.0	84.0	150
Methanogens[b]	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC	56.0	80.0	140
Total Protozoa[c]	GCTTTCGWTGGTAGTGTATT	CTTGCCCTCYAATCGTWCT	55.0	84.2	223
Anaerobic Fungi[d]	GAGGAAGTAAAAGTCGTAACAAGGTTTC	CAAATTCACAAAGGGTAGGATGATT	62.0	83.8	120

[a](Maeda *et al.*, 2003)

[b](Sylvester *et al.*, 2004)

[c](Denman & McSweeney, 2006)

[d](Denman *et al.*, 2007)

2.10 Molecular Techniques: Ion Torrent Next Generation Sequencing

The profile and structure of the rumen bacterial community was determined via sequencing of the 16s ribosomal subunit by Ion Torrent next generation sequencing technology.

2.10.1 Co-Extraction of DNA and RNA

DNA and RNA was co-extracted from samples using extraction method by Griffiths *et al.* (2000). Previously freeze-dried samples were placed into a pestle and mortar and ground into a fine homogenous powder under liquid-N. Approximately 75 mg of powder was weighed into 2 ml micro centrifuge tubes (Sarstedt, Nümbrecht, Germany) along with two titanium beads. To tubes, 500 µl of lysis buffer (Table 2.10) and 500 µl of phenol chloroform isamyl alcohol (25:24:1) pH 8 was added and samples were bead beaten in a Mini-Beadbeater 96 (BioSpec Products, Oklahoma, USA) for 30 seconds.

Table 2.10: Lysis buffer for co-extraction of DNA and RNA (Griffiths, et al. 2000)

Chemical	Quantity
10 % (w/v) Cetrimonium bromide	100 ml
0.7 M Sodium chloride	100 ml
0.1 M Potassium phosphate buffer pH 8	100 ml

Tubes were centrifuged at 16,000 x g at 4°C for 5 minutes and the aqueous phase extracted, volume noted and placed into a clean micro centrifuge tube (Sarstedt, Nümbrecht, Germany). An equal volume of chloroform iso-amyl alcohol 24 : 1 to extract was added to tubes, vortexed and centrifuged at 16,000 xg at 4°C for 5 minutes. The aqueous phase was removed, volume noted and transferred into clean 1.5 ml microfuge tube. Nucleic acids were precipitated out of solution by adding 2 times the volume of extract of polyethylene $glycol_{6000}$-1.6 M sodium chloride and tubes were incubated at room temperature for 2 hours.

Post precipitation, tubes were vortexed and centrifuged at 18,000 xg at 4°C for 10 minutes. The supernatant was discarded leaving a pellet and 500 µl of 70 % ice cold ethanol was added. Tubes were vortexed and centrifuged at 17,000 xg at 4°C for 5 minutes. Supernatant was discarded and the pellet was air dried at room temperature for 30 minutes. Pellets were re-hydrated in 20 µl of Tris-EDTA buffer pH 7.4 incubated on ice for 20 minutes before quantification as described section 2.10.2. Samples were stored at -80°C until later analysis.

2.10.2 Quantification of Nucleic Acids by Fluorescence

Nucleic acids were quantified using a Qu-bit fluorimeter (Invitrogen, ThermoFisher Scientific, New Hampshire, USA) as opposed to a spectrophotometer as described in section 2.9.3. Extracts contained both DNA and RNA therefore quantification of DNA and RNA would not be reliable by a spectrophotometer due to DNA and RNA being read at the same wavelength (260/280 nm). The fluorimeter involves the use of fluorescent probes which bind to nucleic acids therefore DNA and RNA were assigned different colours enabling them to be quantified.

DNA and RNA present within samples was quantified using DNA and RNA broad range kits (ThermoFisher Scientific, UK). A master mix of 1 part fluorescent dye probe to 199 µl buffer solution was made up for DNA and RNA assays and 199 µl of master mix added to 1.5 ml Qu-bit tubes (Invitrogen, ThermoFisher Scientific, New Hampshire, USA) along with 1 µl of sample. Tubes were vortexed and placed immediately into the Qu-bit and the concentration of nucleic acids was measured via emission at 260 nm.

2.10.3 Polymerase Chain Reaction DNA

Amplification of the V1/V2 hypervariable region of the 16S rRNA gene was carried out using PCRBio HiFi Polymerase kit (PCRBiosystems, London, UK). One micro litre of sample was added to 0.2 ml PCR tubes along with 1 µl of 27F forward primer (50 pM) (Eurofins Genomics, Kentucky, USA) linked at the 5' end to an IonTorrent adapter-A sequence (CCATCTCATCCCTGCGTGTCTCCGAC), a TCAG key and a IonXpress Barcode, with the barcode unique to the sample.

A master mix was made up of 5 µl PCRBio HiFi buffer, 1 µl PCRBIO HiFi Polymerase (2 /µl), 0.2 µl 357R reverse primer (Eurofins Genomics, Kentucky, USA) linked at the 5' end to an Ion Torrent B-adapter sequence (CCTCTCTATGGGCAGTCGGTGAT), 17.55 µl of PCR grade water per sample, with 23 µl added to each sample tube. Where DNA samples wouldn't amplify, 2.6 µl of 20mg/ ml molecular grade bovine serum albumin (New England Biolabs® Inc, Massachusetts, USA) was added to each sample and volume of water in master mix reduced to achieve a 25 µl reaction as described by Farell & Alexandre (2012).

Tubes were loaded into a T100™ Thermal Cycler (BioRad, UK) and run for 95 ^{o}C for 1 minute followed by 30 cyles of 95 ^{o}C for 0.15 minutes, 64.2 ^{o}C for 0.15 minutes of 72 ^{o}C for 0.15 minutes followed by 72 ^{o}C for 1 minute and cooling infinitely at 4 ^{o}C. Samples were stored at -20 ^{o}C until further analysis.

2.10.4 Polymerase Chain Reaction RNA

RNA was first transcribed into cDNA using UltraScript Reverse Transcriptase kit (PCRBiosystems, London, UK) with the specific primer 357R (CCTCTCTATGGGCAGTCGGTGATCTGCTGCCTYCCGTA, Eurofins Genomics, Kentucky, USA) which linked at the 5' prime end to the Ion Torrent B adapter sequence (CCTCTCTATGGGCAGTCGGTGAT).

A master mix was made consisting of, 4 µl buffer, 1 µl of Ultrascript, 2 µl 10 pM 357R Primer and 12 µl molecular grade water per sample. Nineteen microlitres of master mix solution was transferred into individual 0.2 ml PCR tubes (Thermo Fisher Scientific, New Hampshire,) along with 1 µl of extracted sample. Tubes were loaded into a T100^{TM}Thermal Cycler (BioRad, California, USA) and run at the manufacturers recommended settings of 42^{o}C for 30 minutes, 85^{o}C for 10 minutes and cooling infinitely at 4^{o}C. Samples were then stored at - 20^{o}C until further analysis.

Once reverse transcribed, cDNA was amplified by Polymerase Chain Reaction (PCR) using PCRBio HiFi Polymerase kit (PCRBiosystems, London, UK). One micro litre of sample was added to 0.2 ml PCR tubes along with 1 µl, 50 pM of 27F forward primer (Eurofins Genomics, New York, USA) linked at the 5' end to the Ion Torrent A-adapter sequence (CCATCTCATCCCTGCGTGTCTCCGAC), a TCAG key and an IonXpress Barcode. A master mix was made up of 5 µl PCRBio HiFi buffer, 1 µl PCRBIO HiFi Polymerase (2 /µl), 0.2 µl p1 reverse primer (Invitrogen, Thermo Fisher Scientific, New Hampshire, UK) of compliment to the adapter sequence attached to 357R, 17.55 µl of PCR grade water per sample with 23 µl added to each sample tube.

Tubes were loaded into a T100^{TM} Thermal Cycler (BioRad, California, USA) and run for 95^{o}C for 1 minute followed by 30 cyles of 95^{o}C for 0.15 minutes, 64.2^{o}C for 0.15 minutes of 72^{o}C for 0.15 minutes followed by 72^{o}C for 1 minute and cooling infinitely at 4^{o}C. Samples were then stored at -20^{o}C until further analysis.

2.10.5 Gel Electrophoresis

To determine if the amplification of the 16S rDNA subunit by PCR was a success, rDNA was detected via gel electrophoresis and visualisation under UV light. A 1 % agarose gel was made by adding 0.5g of agarose (Bioline, London, UK) to 50 ml of 1x Tris-acetate-EDTA (TAE) buffer solution. The solution was heated to dissolve the agarose, before allowing to cool until tepid. Once cool, 0.5 µl of gel red nucleic acid stain (Biotium, California, USA) was added to the solution, mixed and poured into a cast and left to set for approximately 20 minutes at room temperature.

The gel was placed into a horizontal Mini-Sub® Cell GT gel electrophoresis cell (BioRad, California, UK) and loaded with 0.5 µl of 1 kb HyperLadder™ (Bioline, London, UK). Samples were mixed with 1x loading dye (0.25% orange G 30% w/v sucrose in water), 2 µl sample : 3 µl loading dye and 4.5 µl of sample was loaded individually into wells adjacent to the ladder.

The gel was run at 75V for 10 minutes and then at 85V until bands had migrated approximately ¾ of the way down the gel. The gel was removed from the electrophoresis cell and imaged under UV light in a GelDoc™ XR+ (BioRad, California, UK) for 16S rDNA bands. A negative control (sample substituted with water) was run on each gel to check for contamination of PCR reagents.

2.10.6 Purification of Polymerase Chain Reaction Products

PCR products were purified using Agencourt AMPure XP solid-phase reversible immobilization paramagnetic bead technology (Beckman Coulter, California, USA) to remove short fragments of DNA, dNTPs and any residual solutions belonging to the amplification procedure. This technology involves the binding of fragments of DNA to magnetic beads in the presence of PEG. The larger the fragments the greater the charge therefore the greater the affinity to bind to the magnetic beads, however the length of the fragments attached is dependent on the concentration of PEG.

To each µl of PCR product, 1.6 µl of Ampure beads were added. Tubes were incubated at room temperature for 10 minutes before being placed on a Dynamag- 96 side plate with magnets attached (Life Technologies, Thermo Fisher, New Hampdhire, USA) at room temperature for 5 minutes. The supernatant containing short fragments of DNA, dNTPs and residual solution from the PCR procedure was discarded leaving a pellet containing long fragments of DNA attached to the Ampure beads. The pellet was washed twice by re-suspension in 200 µl of 70 % ethanol and left on the magnet for 2 minutes. The pellet was then left to air dry for 2 minutes before being re-suspended in 20 µl of 1x TE buffer solution. Tubes were left on the magnet for a further 2 minutes and the supernatant containing the purified DNA removed and placed into a new 0.2 ml PCR tube. The concentration of nucleic acids was then determined via spectrophotometry as described in section 2.9.3.

2.10.7 Library Preparation, Quantification and Analysis

Based on the concentration of purified PCR products a library was prepared by pooling all samples. Library preparation was based on the weakest sample concentration at a maximum volume of 10 µl. Samples were pipetted into a 1.5 ml microfuge tube and stored at –20^{o}C until later analysis. A subsample of the library was subjected to a final clean-up, to remove fragments < 200 and > 700 base pairs (bp) in length.

Undesired lengths (< 200 and > 700 base pairs bp) of DNA were removed using Agencourt AMPure solid-phase reversible immobilization paramagnetic bead technology (Ampure XP, Beckman Coulter, California, USA) as described in section 2.10.6, via the double size selection technique with modification (Quail *et al.*, 2009). Ampure beads were calibrated in the lab using a 100 bp DNA ladder (PCR Biosystems, Pennsylvania, USA) and the protocol modified in accordance to bead calibration. A 100 bp DNA ladder was used for calibration as the ladder would clearly portray lengths of DNA sequentially at 100 bp in length, therefore it would be easy to identify the concentrations of Ampure bead solutions required to remove fragments of DNA < 200 bp and >700 bp in length.

Step one involved the removal of DNA fragments of < 250 bp. A 50 µl sub sample of the library was placed in a 1.5 ml tube along with 0.65x Ampure XP solution as determined via the calibration procedure. The tube was incubated at room temperature for 5 minutes before being placed on a Dynamag magnetic rack (Invitrogen, Thermo Fisher, New Hampshire, USA) until the solution went clear leaving a pellet containing long fragments of DNA of >250 bp. The supernatant containing short fragments of DNA was removed and discarded, and the pellet washed twice with 200 µl of 70% ethanol (fresh) for 2 minutes. The tube remained on the magnet and the pellet was left to air dry for 2 minutes. The tube was removed from the rack and 60 µl of TE was added and mixed with the pellet, the tube was then incubated at room temperature for 5 minutes before being placed back on the rack until the solution went clear and a pellet formed. The supernatant containing nucleic acids was removed and placed into a new tube leaving the pellet of Ampure beads behind for discarding.

Step two involved the removal of fragments > 700 bp. Approximately, 30 µl of supernatant from the first step was placed into a new 1.5 ml microfuge tube along with 0.475x Ampure XP solution as identified by the calibration procedure. The tube was incubated at room temperature for 5 minutes before being placed on a magnetic rack until the solution went clear. The supernatant containing nucleotides of < 700 bp were removed and transferred into a new tube and the pellet containing nucleotides of > 700 bp and the Ampure beads discarded. A 44.25 µl sub sample of the supernatant was taken and placed in a new tube along with 0.565x Ampure XP solution creating a final concentration of 1.04x Ampure XP in solution, the tube was then incubated at room temperature for 5 minutes. The tube was then placed on a magnetic rack until the solution was clear and a pellet formed containing nucleic acids between 250 – 700 bp in length. The supernatant was discarded, and the pellet retained. The pellet was left on the rack and washed twice with 200 µl of 70% ethanol (freshly prepared) for 2 minutes before being left to air dry for a further 2 minutes. The tube was removed from the rack and 40 µl of TE was added, mixed with the pellet and left to incubate at room temperature for 5 minutes before being placed back on the rack until the solution went clear and a

pellet formed. The supernatant was removed, placed into a new tube and stored at –20^{o}C until analysis.

The library was analysed via chip based capillary electrophoresis using a Bioanlayser 2100 (Agilent Technologies, USA) to ensure all short and long fragments had been removed and to determine the molar concentration of the library for dilution to 50 pM in preparation for sequencing.

2.10.8 Emulsion PCR, Enrichment and Chip Loading

Emulsion PCR, enrichment and chip loading were conducted by use of an IonChef (Life Technologies, Thermo Fisher, New Hampshire, USA). Twenty-five µl of diluted library was subjected to emulsion PCR, in which nucleotides were bound to beads surrounded in an oil droplet and amplified. The bead nucleic acid complex was then subjected to enrichment using magnetic streptacation to remove beads and the oil droplet surrounding the nucleotides. The library was then loaded onto a 316v2 (100 Mbp) microchip (Life Technologies, Thermo Fisher, New Hampshire, USA). Chips were then placed into the Ion torrent Personal Genome Machine (PGM) (Life Technologies, Thermo Fisher, New Hampshire, USA) and nucleic acids were sequenced.

2.10.9 Bioinformatics Pipeline

The pipeline for sequence data analysis into an operational taxonomic unit (OTU) table was written and performed by post-doc within the lab, Andrew Detheridge.

Binary Alignment Maps (BAM) produced by the IonTorrent were converted into FASTQ format, quality checked and demultiplexed using MOTHUR version 1.31.2 (Schloss *et al.*, 2009). Sequences of < 100 bp with an average Phred score < 20, mismatching barcodes and mismatching sequence primers were all removed and as suggested by Tedersoo *et al.* (2010) sequence files were de-replicated and singletons discarded. Sequence data was then clustered into OTU, set at 97 % clustering with clusters containing less than 2 sequences discarded using UPARSE (Edgar, 2013). Taxonomy was assigned to each OTU using the Naïve Bayesian Classifier (Wang *et al.*, 2007) against an adapted version of the rumen database project (RDP) for bacteria with a 0.6 cut off for bacterial sequences. Where genus was not assigned by the classifier, but family and order were, an OTU was assigned. Relative abundance of taxa was calculated in Excel version 2016 (Microsoft, Washington, USA) by dividing the number of reads in each taxonomic unit by the total number of bacterial reads. Any non-bacterial taxa were reported separately.

2.11 Metabolic Function of Bacteria

Prediction of the potential metabolic function of bacteria was investigated using CowPI (Wilkinson *et al.*, 2018) a rumen version of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 165 (PICRUSt) (Langille *et al.*, 2013). Briefly, DNA and RNA samples were separated into two datasets with each set normalised to the lowest sequence count to avoid bias. Normalised OTU tables and FASTA files containing OTU sequences were uploaded into Galaxy along with the pre-calculated 16S and keg orthologue (KO) files supplied by CowPi. OTUs in the DNA and RNA datasets were screened against OTUs in the pre-calculated 16S CowPI file which contained 16S sequences identified in the Global Rumen Census Project and Hungate 1000 project. Using pre-calculated CowPI KO file the metagenome was predicted via the frequency of KO as determined in the Hungate 1000 project. The resultant gene count data was then normalised by copy number producing information on the potential genomic and metabolic function of the bacterial community.

Chapter 3 Effect of Low Moisture Feed Lick Supplementation on the Performance of Commercial Breeding Ewes in the Uplands

3.1 Introduction

Sheep farming is a traditional occupation in many parts of rural Britain with farming stratified into the production systems; breeding for finished lamb production, breeding for store production, store finishing and breeding stock production. Production systems are predominantly extensive and are largely dependent on land topography and include the grazing of marginal land. The UK sheep flock comprises of over 33 million sheep of which over 16 million are estimated to be breeding ewes (NSA, 2015). The UK is the 6th largest global producer and the 3rd largest consumer, importer and exporter of ovine meat (AHDB. & IMS., 2016) and is estimated to employ 34,000 people on farms and 111,405 people in related industries providing 291.4 million pounds to the economy (NSA, 2020).

Nutrition is an integral part of livestock production systems however the cost of feed contributes to a large proportion of on farm variable costs. Forage is the cheapest feed resource available for ruminants, however there are inconsistencies in the nutritional quality and availability of pasture throughout the grazing season and at times forage alone may be insufficient in quantity and nutritional quality to achieve production demand. Low moisture feed licks (LMFL) retailed as forage balancers and are a popular method of supplementation for grazing ruminants as described in Chapter 1 section 1.6.3. Licks are placed out at pasture for self-regulatory consumption providing additional nutrition to the diet in the form of energy, protein, vitamins and minerals. Although research in the literature is limited, previous research has demonstrated ruminants supplemented with LMFLs at grazing to have positive effects on animal performance in terms of live weight gain (Hart & Newbold, 2015) body condition and blood metabolic status (Cabiddu *et al.*, 2014).

3.2 Chapter Aims

This chapter is a preliminary study which aims to investigate the use of LMFL supplementation in a commercial environment. In this small-scale study, the effect of LMFL supplementation was investigated on the performance of breeding ewes and their lambs on a commercial farm practicing extensive grazing in the uplands. A commercial farm was chosen to observe the effects of the LMFL supplement as it would be utilised in farming enterprises in the UK. The upland was chosen as a location due to a harsher climate and a poorer nutritional quality of grazing and availability in comparison to the lowland, therefore it was thought that supplementation would be required in this environment. Moreover, supplementation at grazing is typical practice in the uplands due to the harsh climate and forage quality and availability being limited at times.

The utilisation of LMFLs was investigated by measuring the consumption of the LMFL by breeding ewes from tupping to parturition in conjunction with climate, sward growth and sward dry matter. The performance of breeding ewes was investigated by recording live weight each month from pre-

tupping to weaning, recording the number of lambs per ewe at scanning and measuring the duration of the lambing period as an indirect indicator of reproductive performance. The performance of lambs was investigated by measuring live weights each month until weaning and recording the time taken to reach commercial viability for slaughter and the subsequent lamb carcass quality.

3.2.1 Hypotheses

The hypothesis of this chapter are detailed below:

H_1: Consumption of the LMFL by the group of ewes will increase over the winter months

H_2: Ewes supplemented with the LMFL will have greater live weights in comparison with ewes receiving no supplement

H_3: Ewes supplemented with the LMFL will have a greater number of lambs *in-utero* at scanning in comparison with the control group

H_4: Lambs whose dams are supplemented with the LMFL will reach slaughter weights sooner in comparison with the control group

H_5: Lambs whose dams are supplemented with the LMFL will have better carcass grades and in comparison with the control group

3.3 Experimental Setup

3.3.1 Experimental Location, Animals and Treatments

The trial was conducted on a commercial upland farm in the Cambrian Mountains (Pontatfynach, Aberystwyth) approximately 300 metres above sea level. The trial started on the 10th October 2018 and finished on the 21st December 2019 with tupping commencing on the 23rd of October 2018 approximately 14 days post the initial start date of the trial. The trial followed the performance of breeding ewes pre-tupping to weaning and their lambs until finishing with investigations into carcass characteristics and quality. No invasive procedures were carried out in this trial and all medicinal and animal husbandry procedures were carried out by the farm.

Eighty Texel x (Charollais, Blue Faced Leicester, Speckeld Faced Welsh Mountain, Welsh Mountain) and 20 Speckle Faced Welsh Mountain ewes (mean ± stdev live weight: 64 ± 6.5 kg; 3.5 years ± 1.5) were recruited onto the trial and split into two groups of 50 at random, with no replication groups within each group. For management purposes, ewes were given a painted smit mark (black = control group, green = LMFB group) on their necks for identification of each ewe to each group. It was later found that one of the ewes in the control group was a mule therefore this ewe was removed from the trial.

Groups were allocated to adjacent fields of permanent grazing at an initial average sward height of approximately 3.25 ± 0.71 cm. Fields were last re-seeded with Special POCHON Persistent High Clover Long Ley Mix (Wynnstay, Llansantffraid, UK) at 13.6 kg/ acre in the early 2000's. Groups were assigned to one of two dietary treatments, 1) Control, grazing; 2) Treatment, grazing plus LMFL supplementation. The LMFL provided was Crystalyx® Extra High Energy as described in Chapter 2 section 2.2.1. This specific LMFL is one of the most popular licks of the Crystalyx® range and is specifically formulated to cater for the production needs of breeding ewes, rams and growing lambs throughout the shepherding calendar.

Two 22.5 kg tubs of the LMFL were placed out at pasture at opposite ends of the field for ewe consumption for free access. Tubs were immediately replaced when empty. During the winter months (23rd January–18th March) when grazing was limiting in availability both groups were supplemented with grass silage made on farm that summer. During the lambing period both groups were supplemented with approximately 0.14 kg per head per day of Ewe Lac 18 Rolls (Bibby Agriculture, UK) for approximately 50 days in support of lactation. It was decided to not remove this period from the data set due to all ewes receiving the additional nutrition. Chemical analysis of the silage and ewe rolls was conducted as described in Chapter 2 section 2.6 to determine the dry matter, organic matter, neutral detergent fibre, acid detergent fibre, crude protein and ash content (Table 3.1).

Table 3.1: Chemical analysis of supplementary feeds, grass silage and ewe rolls fed to ewes over the winter grazing period

	Grass Silage	Ewe Rolls*
Dry Matter (g/ kg)	397	944
Organic Matter (g/ kg DM)	903	901
Neutral Detergent Fibre (g/ kg DM)	681	472
Acid Detergent Fibre (g/ kg DM)	407	248
Crude Protein (g/ kg DM)	139	194
Ash (g/ kg DM)	96.7	98.8

*Ewe Lac 18 Rolls (Bibby Agriculture, Carmarthen, Wales)

3.3.2 Farm Production System

The farm production system was extensive with an outdoor lambing system. Full details of the shepherding calendar can be found in Table 3.2. Approximately 14 days after the initial start date of the trial (23rd October) one Texel ram joined each group followed by a second Texel ram 14 days later. The tupping period lasted for 98 days allowing for approximately 4.6 oestrous cycles. Ewes were gathered at the end of January (29th January) and underwent ultrasound scanning to determine pregnancy and the number of expected lambs per ewe. Rams were removed from groups at scanning along with any barren ewes. In January a copper bolus was given to all ewes to account for historic cases of swayback in lambs, however this was not based on any recent blood or hepatic analysis. In addition, the LMFL did not contain any copper. Lambing commenced from the 18th March–6th May. Any non-rearing ewes as a result of complications pre and post-partum were removed from the trial. Lambs were weaned from their dams at the end of August. Lambs were kept in their groups and LMFL supplementation continued until finishing (August-December).

Table 3.2: Details on the key events, additional nutrition and health plan of breeding ewes and their lambs grazing in the uplands

	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Key Events															
Tupping															
Scanning															
Lambing															
Shearing (E)															
Weaning															
Lamb Finishing															
Additional Nutrition															
Winter Silage (E)															
Ewe Rolls (E)															
Health Plan															
Copper Supplement															
Heptavac Booster															
Anthelmintics*															
Insecticide Pour On**															
Dagging (E,L)															

E = Ewes, L = Lambs

*Combinex (E), Albex (L) Cydectin (L)

**Crovect (E,L), Clik (E,L)

3.3.3 Grazing Measures

Exclusion cages (0.5 m x 0.25 m) were placed out at pasture for measuring monthly grass growth (cm). Cages were thrown in the field to randomly select the cage location and held securely in place with tent pegs. The initial sward height within the cage was measured by placing a board on top of the swards and measuring the height with a ruler at 5 random locations (Figure 3.1). Each month cages were removed and sward height measured to determine the average monthly sward growth rate. Sward samples were taken monthly by walking across the field in a "W" shape randomly selecting samples and cutting swards at their base with scissors. Samples were transported back to the laboratory on Penglais campus at Aberystwyth University for freezing at -20^{o}C until later analysis to determine the dry matter content of samples as described in Chapter 2 section 2.6.1.

Figure 3.1: Measurement of sward height and an example of the exclusion cage used

3.3.4 Climate Measures

Information on the weather such as air temperature (°C), rainfall (mm), wind speed (m/s) and solar radiation (W/m^2) were obtained from Parc Newydd weather station located at Aberystwyth University's Upland Research Centre in Cwmystwyth, approximately 320 m above sea level and 6 miles away from the trial location.

3.3.5 Ewe Performance Measures

Ewe performance was measured from tupping to weaning (October-August), with analysis of the data conducted from tupping to parturition (October-March) and post-partum to weaning (May-August). It was decided to split ewes into groups; single bearing and twin bearing due to the difference in weight between the two conditions. Moreover, if one group had a greater number of twin bearing ewes this may bias the mean live weight of the group.

Ewes were gathered from grazing once a month, brought indoors, their electronic identification (EID) tags scanned using EID reader (Tru-Test, Auckland, New Zealand) and their live weights recorded in a portable weigh crate linked to a weigh head (Tru-Test, Auckland, New Zealand) connected to the EID reader via Bluetooth. Scanning took place on the 29th of January using a portable ultrasound scanner by experienced scanner Janet Downing, to predict the number of lambs a ewe was pregnant with. During the last month of gestation (14th March) ewes were given a unique ID sprayed onto their wool with stock marker. Ewes expecting singles were given a letter and ewes expecting twins a number. Ewes in the control group were marked with a blue marker and ewes in the treatment group were marked with a red marker for ease of identification. When ewes had given birth to lambs, lambs received the same ID as their dam sprayed onto their wool using stock marker to identify ewe to lamb.

3.3.6 Lamb Performance Measures

It was not possible to collect individual lamb birth weights within 14-48 hours of birth due to the nature of the production system, however lamb live weights were recorded each month from May until August (weaning) as described in section 3.3.4. Within 24 hours of birth, lambs were assigned with the same ID and colour as their dams as described in section 3.3.4. Lamb live weights were recorded before slaughter to determine finishing live weight. Before going off to slaughter lambs were given a unique EID tag which was scanned and recorded as described in section 3.3.4 for traceability in correspondence with kill lot data. Lambs were transported to the abattoir (Dunbia, Llanybydder, Wales) when the farm considered them as commercially viable based on physical appearance, body conformation and live weight (35-40 kg). Lambs were entered into one of two carcass specifications 1) Heavy carcass specification with a target dead weight of 17.9 – 22 kg; 2) Small carcass specification with a target dead weight of 8 – 14.9 kg. Kill lot details included information on the carcass grade awarded, the carcass hot weight and an estimate of the cold weight based on a rebate of 0.5 kg. Carcass grading was based on the lean and fat content of carcasses by the EUROP scale as described by AHDB (2016).

3.4 Statistics

The starting live weights of ewes, lamb finishing weights, cold carcass weights and the killing out percentages of lambs were analysed via one-way analysis of variance (ANOVA) using Genstat 19th edition. Significance was regarded as $p<0.05$.

Due to uneven sample sizes between groups and within groups, repeated measures ANOVA could not be performed on the live weights of ewes and lambs. Therefore, live weight was analysed via repeated

measures residual maximum likelihood mixed model (REML) with a single variate in Genstat 19th edition. Significance was regarded as $p<0.05$. To overcome unbalanced numbers in each sample group the trial should have been split into two separate trials, with trial 1 following ewes from tupping to scanning and trial 2 selecting an equal number of ewes carrying singles and twins and following them and their lambs until weaning.

Scanning data and carcass grades were categorised into a contingency table using the pivot table function in Microsoft Excel and analysed using Chi squared analysis. Chi squared analysis for the scanning data was carried out on the count data for single and twin bearing ewes, due to no differences in the number of ewes scanned as empty between the two groups. In addition the sample size of each group was small (n=49 -LMFL group and n= 50 +LMFL group). Significance was regarded as $p<0.05$.

3.5 Results

3.5.1 Low Moisture Feed Lick Utilisation by the Group of Ewes

The utilisation of the LMFL by the group of ewes, climate and sward characteristics were measured from tupping to parturition (October-March). It was thought that the availability of forage and weather conditions over the winter months could influence the utilisation of the LMFLs by the ewes. The winter was relatively mild (Table 3.3). January was the coldest month with the least solar cover and rainfall in comparison with other months. February was a relatively mild month with an average daily temperature of 7°C and increased solar radiation suggesting the initiation of possible grass growth on entering into the spring where March exhibited the highest rainfall out all months. There were no significant correlations detected between sward height and climate details.

Sward height was greatest in October and lowest in February (Table 3.4). Sward height decreased each month up until March where it began to increase in height. Sward growth was variable with growth lowest in January and the greatest in November and March. The dry matter content of sward samples were similar to values published by Ewing (2016), however the dry matter content of forage increased in February and was still high in March.

Table 3.3: Climatic conditions from October to March

	October	November	December	January	February	March
Temperature (^{o}C)	8.7 (4.9-12.2)	7.6 (5.2-9.8)	7.2 (7.1-8.8)	3.8 (1.2-5.7)	7.0 (3.2-10.3)	6.4 (3.3-9.3)
Wind Speed (m/s)	2.4 (0.7-4.7)	3.49 (1.4-6.1)	3.1 (2.6-5.3)	2.4 (0.7-4.8)	3.1 (1.1-5.5)	3.4 (1.1-6.3)
Radiation (W/m^2)	67.4 (0-440.9)	36.7 (0-259.9)	14.6 (0-128.2)	26.1 (0-188.8)	65.1 (0-404.4)	87.8 (0-498.6)
Total Rainfall (mm)	125.0	124.7	191.2	77.9	135.9	322.1

Values are means (minimum-maximum)

Table 3.4: Sward characteristics from October to March

	October	November	December	January	February	March
Average sward height (cm)	4.50	2.75	2.00	2.87	1.00	2.50
Average sward growth (mm/day)	/	0.454	0.263	0.326	0.667	0.536
Sward dry matter (g/ kg)	210.4	182.6	199.7	203.3	300.5	268.9

Variation resided in the monthly consumption of the LMFL by the group of ewes. Consumption increased up until December where the group was consuming approximately 43.3 g / day (Figure 3.2). There was a decline in consumption in January to approximately 27.0 g/ day possibly due to addition of winter feeding of silage, however consumption increased in February to 45.8 g / day. Lick intake decreased in March to approximately 39.2 g/ day which may have been due to ewes preparing for lambing or due to early spring grass beginning to come through. However, it must be noted that there were no replicate groups within the +LMFL group, therefore utilisation of the LMFL is based on one group alone and doesn't take into account factors such as animal behaviour, for example hierarchal structure on the consumption of the LMFL.

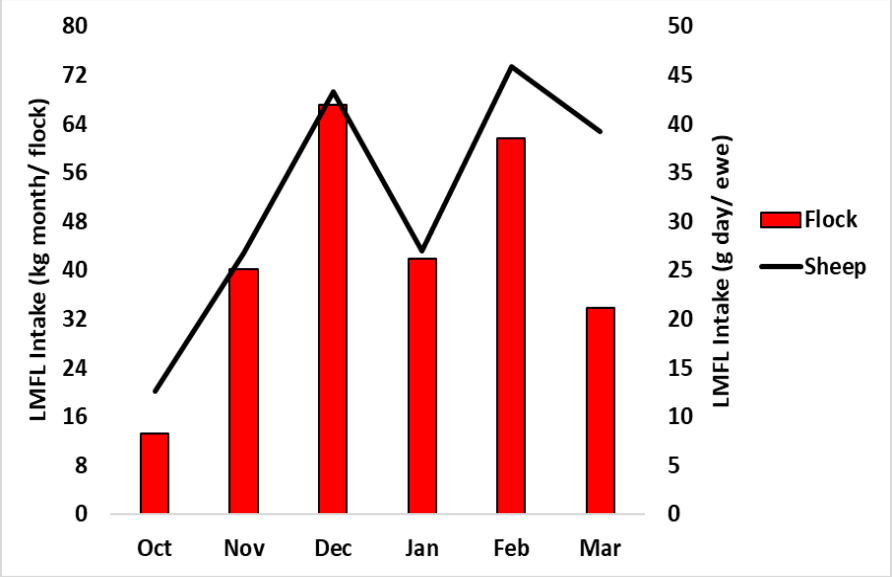


Figure 3.2: Estimated low moisture feed lick (LMFL) consumption of a group of ewes over the winter months (October-March expressed on a flock basis (kg/ month) and on an estimated individual ewe (g/ day) intake basis. Number of ewes was 50 from October – January after January number ewes were 38.

3.5.2 Ewe Performance

Ewes at the start of the trial were perceived to be of an optimal body condition score for tupping (body condition score of 3.7) according to AHDB (2014). ANOVA revealed a significant difference in the live weight of sheep between the two groups ($p=0.034$) at the start of the experiment, with the -LMFL group on average being heavier in comparison with the +LMFL group by 2.8 kg (mean ± stdev live

weight, control group 65.7 ± 6.0 kg; treatment group 62.9 ± 6.8 kg). Therefore, all live weight data in subsequent months were analysed as a percentage of ewe live weights at the start of the trial (Figure 3.3). To overcome this, ewes should have been split into two groups based on their live weights rather than at random. It must be remembered that the number of ewes in each group was 50, which is a relatively low sample size with each ewe accounting for 2% of the group, however this was the maximum number of sheep the farm could provide for the trial. Any data presented on a percentage basis should therefore be reviewed with caution and why figures in the results section at times display actual values in addition to percentages. Throughout the trial ewes were removed at various time points for reasons such as, empty at scanning, non-rearing post-partum due to complications at lambing such as abortion, prolapse, loss of lambs or removal from the group to foster orphan lambs and managerial issues such as drying up or medical treatment (Table 3.5). This resulted in an uneven sample size in groups throughout the trial where 33 ewe in the control group and 42 ewes in the treatment group successfully completed the trial from tupping to weaning. To overcome this the minimum number of animals recruited onto the trial should have been calculated by power calculation as described by Charan & Kantharia (2013) or based on previous studies.

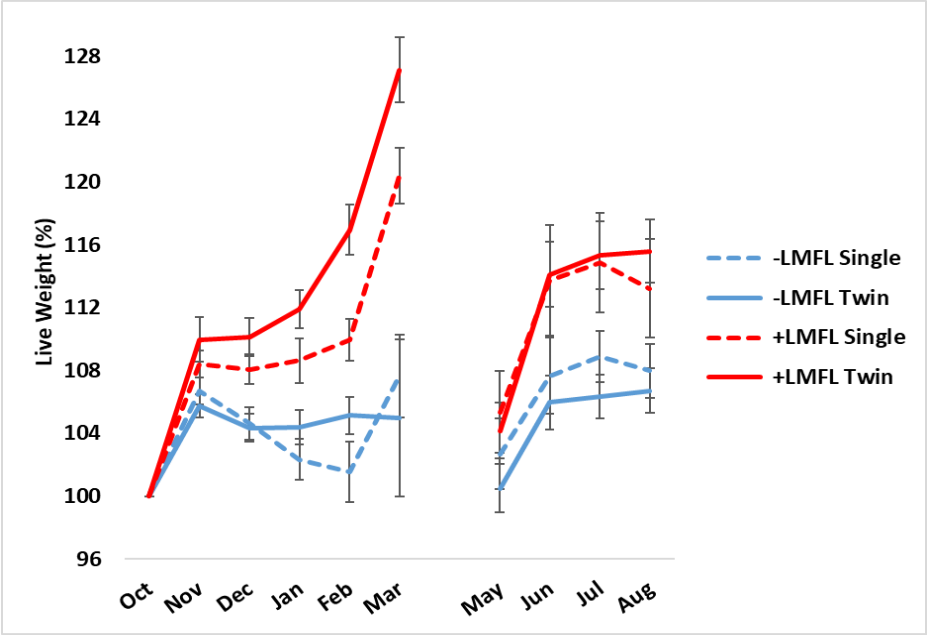


Figure 3.3: The effect of low moisture feed lick (LMFL) supplementation on the live weight gain of ewes carrying single (- -) or twin (-) lambs from tupping to parturition (October-March) and post-partum to weaning (May-August). Where – represents the absence of the LMFL and + represents the presence of the LMFL. Values are expressed on a percentage basis of their initial live weights in October. Values are expressed as means of the groups and error bars are the standard error of difference. No data is displayed for April due to it being the lambing period. Tupping-parturition, supplementation, LMFL $p<0.001$, lamb numbers $p=0.089$, interaction $p=0.312$; post-partum-weaning, supplementation, LMFL $p=0.002$, lamb numbers $p=0.771$, interaction $p=0.654$.

From tupping to parturition (October-March) ewes supplemented with the LMFL displayed greater live weight gains in comparison to the un-supplemented group ($p<0.001$), an observation that was also made post-partum to weaning (May-August) ($p=0.002$). All ewes in each group gained weight from October to November (Figure 3.3). The live weights of single bearing and twin bearing ewes supplemented with the LMFL increased from October to March with an exponential increase in live weight between February and March in accordance with foetal exponential growth. The live weights of the control animals did not follow a similar pattern instead live weight in single bearing ewes decreased from November to February and the live weight of twin bearing ewes was no different from November to March.

Post-partum the live weight of ewes decreased as a result of the birth of the foetus. The live weight of ewes in the control group returned to a similar weight to what was observed at tupping. However the liveweight of the treatment group remained heavier in comparison with weight at tupping. Both treatment groups experienced gains in live weight from May to August with the supplemented group remaining heavier than the control group. There was a tendency for twin bearing ewes to have greater live weights in comparison to single bearing ewes from tupping to parturition (p=0.089). There was no difference between the live weights of single and twin bearing ewes post-partum to weaning. There were no significant interactions between factors.

Table 3.5: Information on the number of ewes per group and reasons for removal from trial

	-LMFL	+LMFL
Ewes in group	49	50
Rearing ewes	33	42
Empty at scanning	4	2
Non-rearing ewes*	4	3
Aborted/prolapse	7	0
Death at lambing	1	3
Management**	2	1

- Absence of the LMFL
+ Presence of the LMFL
* Lamb deaths adoption or orphan lambs
**Removal from trial due to drying up, medical treatment, body condition

Scanning data (Figure 3.4) revealed ewes supplemented with the LMFL to have a scanning percentage of 166%, which was 13.7 % greater in comparison with the control group which had a scanning percentage of 146%. In the control group there was a greater number of ewes bearing single lambs whilst in the treatment group there was a greater number of ewes bearing twins. There was no difference in the number of ewes that scanned as empty in each group. Chi squared analysis revealed there to be a tendency for an association of LMFL supplementation and the number of lambs a ewe scanned for (p=0.078).

Lambing commenced from the 18th March to the 6th May. The treatment group finished lambing on the 9th April giving a lambing period of approximately 22 days, whereas the control group finished lambing on the 6th May giving a lambing period of 49 days. After lambing there were 33 ewes

remaining in the Control group and 42 ewes in the LMFL group, details of what happened to the non-rearing ewes can be found in Table 3.5.

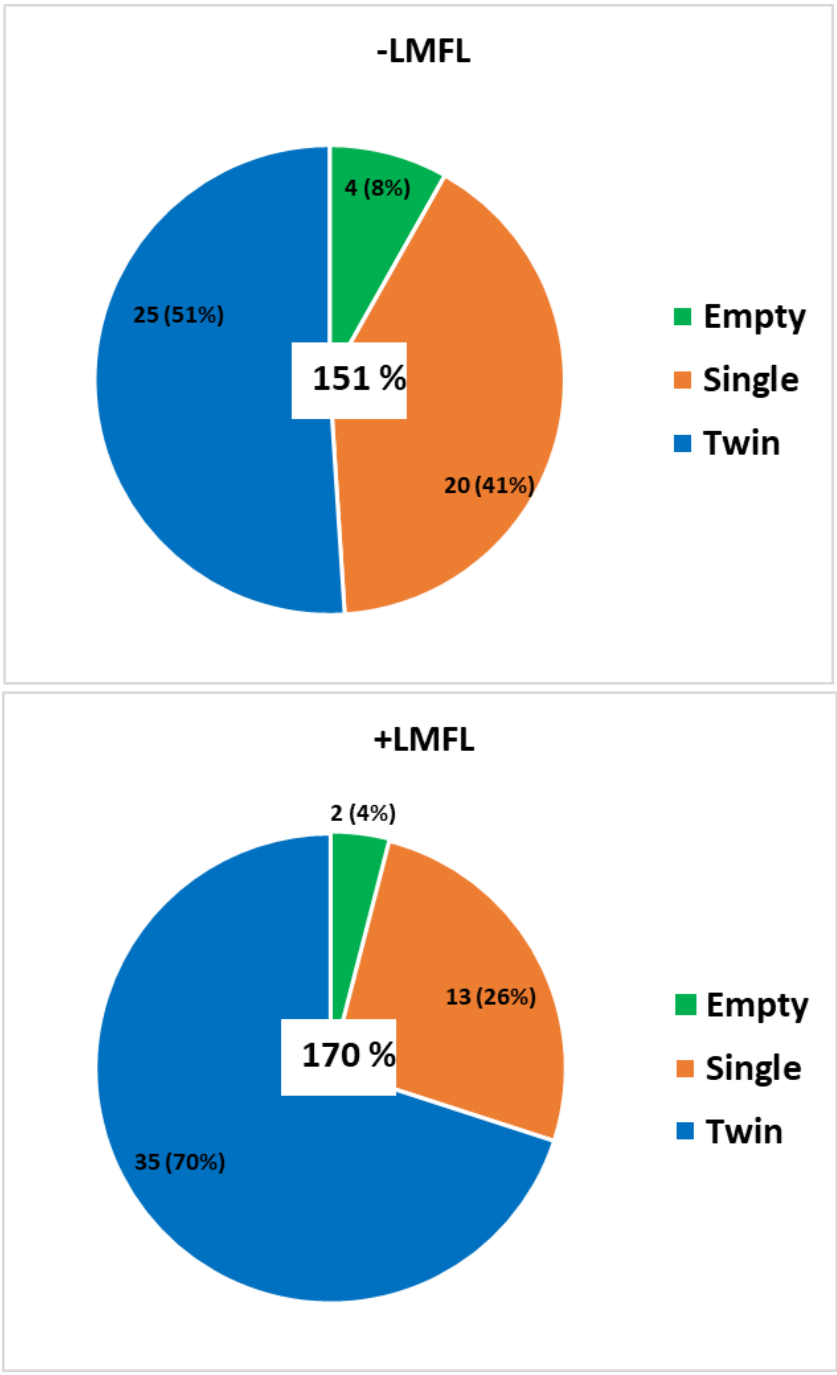


Figure 3.4: Scanning results detailing the number of lambs ewes were carrying in the group supplemented without (-LMFL) and with (+LMFL) the low moisture feed lick. Values are count values and percentage values are in brackets. Percentage values in white boxes are the overall scanning percentage of the group in which the +LMFL group was 12.6% greater in comparison to the -LMFL group (p=0.078).

3.5.3 Lamb Performance and Carcass Characteristics

There were 48 live lambs (24 Male and 24 Female) present in the control group and 65 live lambs (22 Male and 43 Female) present in the supplemented group. This was much lower than expected according to the scanning results (section 3.5.2). Lamb live weights were recorded after lambing had finished (May) for convenience of the farm. Live weights were recorded up until weaning at August. Lambs were not given EID tags until slaughter therefore identification was based on their unique ID marks given as described in section 3.3.5. However, miscommunications at lambing resulted in this system failing and lambs were not able to be identified to their dams or on an individual lamb basis. Gender of the lambs was first recorded during June, therefore the live weights of lambs were analysed collectively within their groups to allow for the inclusion of data from May. Lambs supplemented with the LMFL had greater live weights in comparison with the control group ($p<0.001$) (Figure 3.5). It was not possible to calculate the individual average daily live weight gain of lambs within groups due to lack of identification.

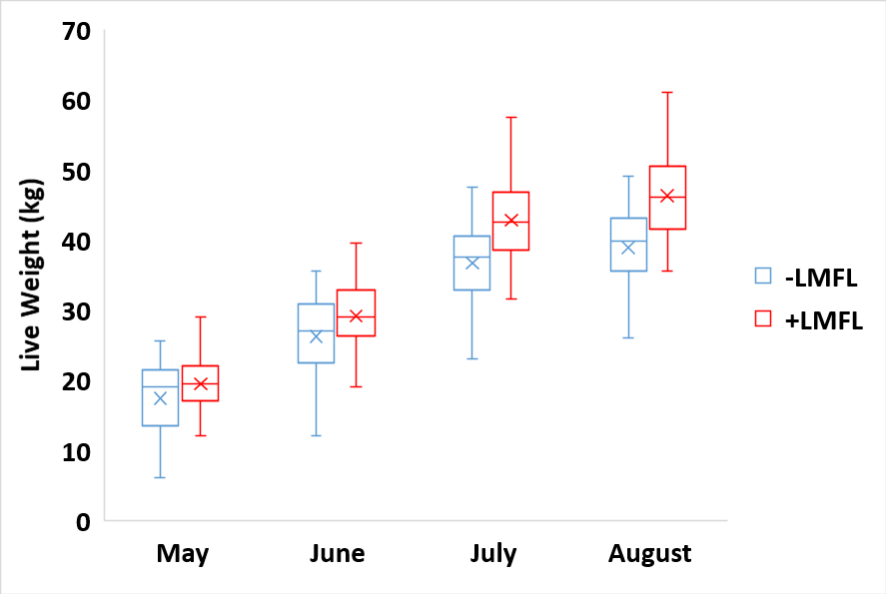


Figure 3.5: Live weights of lambs supplemented without (-) and with (+) the low moisture feed lick (LMFL) from May-August up until weaning. Boxes represent the interquartile range, lines in boxes represent the median, x represents the mean and whiskers represent minimum and maximum values. LMFL $p<0.001$.

All male lambs went for slaughter however a number of ewe lambs were kept as replacements due to the farm operating a closed flock system with the only new blood on farm being from rams. From the control and treatments group 7 and 21 ewes respectively were kept as replacements. From personal communication from the farm a greater number of ewes were kept as replacements from the treatment group as they were of a better confirmation in comparison to the control group. From the control group 41 lambs (24 Male, 17 Female) went off for slaughter and from the treatment group 44 lambs went off for slaughter (22 Male, 22 Female). Variation resided in the date lambs went off for slaughter and the carcass specifications (large carcass/small carcass) lambs were entered into (Table 3.6).

The majority of lambs in both groups went off for slaughter in August and September. Only 3 lambs were remaining in the treatment group in September whereas 11 lambs remained in the control group. All lambs in the control group had gone off for slaughter in October, whereas all lambs in the treatment group had on off for slaughter by December. However, lambs in the control group were entered into the small carcass category in October.

Table 3.6: The number of lambs and gender of lambs sent off for slaughter each month from groups supplemented without (-) and with (+) the low moisture feed lick (LMFL)

Month	-LMFL	+LMFL	Specification
August	21 (12M, 9F)	29 (16M, 13F)	Large carcass
September	9 (6M, 3F)	13 (4M, 9F)	Large carcass
October	11 (6M, 5F)	1 (1M)	Small carcass
November	0	1 (1M)	Large carcass
December	0	1 (1M)	Large carcass

Values are count data
M = male
F = female

Lambs supplemented with the LMFL displayed greater live weights at finishing ($p<0.001$) in comparison with lambs from the control group (Table 3.7). Kill lot data revealed lambs in the supplemented group displayed heavier cold carcass weights ($p<0.001$) in comparison with lambs from the control group. However, it must be reminded that 11 lambs from the control group entered the small carcass specification. There was no difference between the killing out percentage of carcasses from lambs in the control and supplemented group.

Table 3.7: Finishing weights and carcass characteristics of lambs supplemented (-/+) the low moisture feed lick (LMFL)

	-LMFL	+LMFL	sed	p-Value
Finishing weight (kg)	39.3	44.3	0.963	<0.001
Cold weight (kg)	17.2	19.1	0.503	<0.001
Killing out (%)	43.1	42.3	0.704	0.262

Kill lot data revealed that the majority of carcasses were of ideal carcass grading specifications for return (Figure 3.6). Chi squared analysis was carried out on carcass grades with grades low or high (3L, 3H, 4L, 4H) merged into the number category. Chi squared statistic for ewe lambs revealed dietary treatment to be associated with carcass grade (p=0.03) however this was not observed for male lambs. However, this data should be considered as preliminary data as comparisons were not made at a specific age or weight but when lambs were deemed commercially viable for slaughter. Therefore, further research is required to determine the effect of LMFL supplementation on carcass grade and quality.

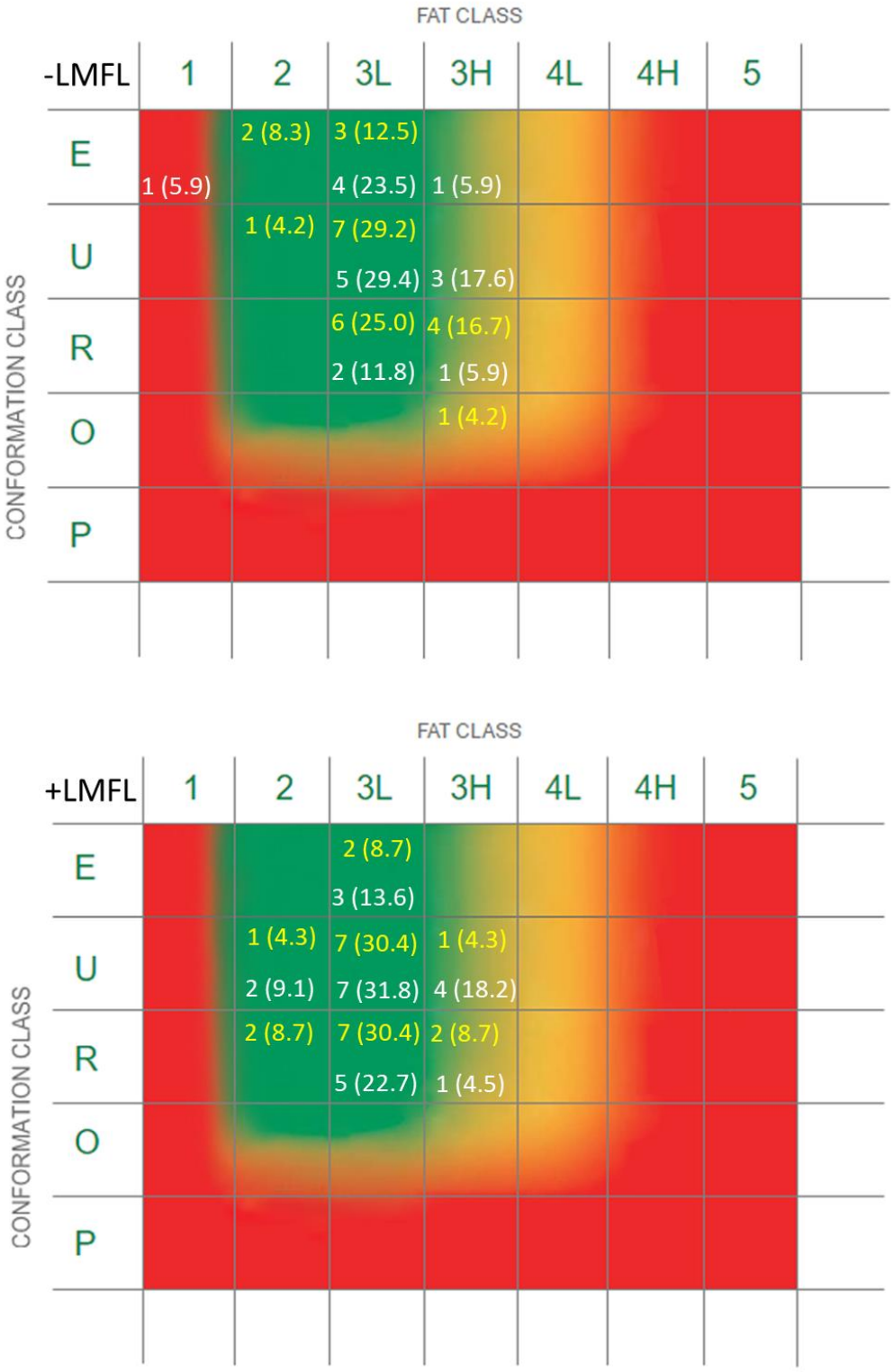


Figure 3.6: Carcass grading (EUROP scale) for lambs supplemented without (-) and with the (+) low moisture feed lick (LMFL). Values are count and (percentage), values in yellow are males and values in white are females. Image of choropleth grid obtained from AHDB (2016).

3.6 Discussion

The main aim of the trial was to observe the effect of LMFL supplementation in a commercial environment investigating, 1) the utilisation of the LMFL by the group of ewes over the winter months; 2) the effect of LMFL supplementation on ewe performance and 3) the effect of LMFL supplementation on lamb performance and carcass characteristics. However, difficulties were faced conducting a controlled animal trial in a commercial environment in terms of controlled measures that could be implicated. In addition, complications occurred when trying to identify lambs to dams. In the aim of preventing infection early on in life ear tags were only given to lambs before going off for slaughter which also eliminates the risk of ear tag loss in the field. To overcome, ewes were given a unique letter or number sprayed onto their fleece with stock marker and their lambs were to be given the same ID for matching lamb to dam. However, miscommunications at lambing resulted in this system not working effectively and as lambs grew the marks tended to fade and stretch so they were unidentifiable. Therefore individual lamb live weight gain could not be calculated. Likewise, the lambing system was an extensive outdoor system in which ewes were lambing over a 10 acre space and post lambing turned out onto a space of 15 acres, therefore it was not possible to record the live weight of lambs soon after birth. Likewise, there was a concern ewes may mis-mother if lambs were disturbed so soon after birth. In addition, the animal trial had a limited number of ewes within each group (n= 49 control group and n=50 treatment group) therefore making any statistical analysis limited as a result of removal of ewes from trial or differences in the number of lambs a ewe was bearing in each group. This resulted in unequal sample sizes within groups and between groups. Therefore, any results in this chapter should be reviewed as preliminary and this trial should be carried out again on a larger scale and with increase control measures.

3.6.1 Low Moisture Feed Lick Utilisation by the Group of Ewes

There is limited research in the literature into the utilisation of LMFLs by sheep grazing in temperate climates such as the UK. Consumption of the LMFL was measured by recording lick consumption in conjunction with climate (temperature, rainfall, wind speed and solar radiation) and grazing measures (sward height, sward growth, dry matter). It was decided to measure the consumption of the LMFL by the group of ewes from tupping to parturition (October-March) because post-partum lambs would be in the field with ewes and it would have been difficult to estimate individual ewe intakes. It was not possible to measure LMFL consumption on an individual ewe basis, therefore consumption was measured by calculating the weight of the block consumed by the group per month and dividing by the number of animals in the group. In addition there were no replicate groups within the supplemented group thereby limiting the results. Taylor *et al.* (2002) highlights that although LMFL

are a convenient method of supplementation on a practical basis for the producer, they are not a reliable method of supplementation in guarantying every animal will consume them, consume a sufficient quantity or frequently consume them. Therefore, results in this study should be viewed as an estimate of consumption rather than an absolute.

LMFL consumption from pre-tupping to parturition (October-March) was estimated to range from 12.6 - 45.8 grams per day per ewe with an average intake of 32.4 g per day across the observation period. This is low in comparison to manufacturers guidelines whom suggest ewes to consume between 40 - 60 g of the lick per day (Caltech-Crystalyx®-UK, 2019). Likewise, Cabiddu *et al.* (2014) observed dairy ewes in the Mediterranean to consume approximately 164 g of LMFL per day 60 days pre-partum and 76 g of LMFL per day 60 days post-partum. Reasons for differences in LMFL consumption may be due to climate in which higher temperatures experienced in the Mediterranean may result in the lick softening and being more readily available for consumption. Likewise, as mentioned previously it was not possible to tell if all ewes consumed the LMFL or how much of the LMFL each individual consumed.

LMFL consumption was lowest in October (12.6 g / ewe / day), this is probably a result of the time taken for ewes to locate the LMFL and due to grazing being available in which the average sward height of the field at that time was estimated to be 4.5 cm. Moreover, the summer of 2018 was "record breaking" with the Met_Office (2019) reporting weather as warm and wet, resulting in silage cut later on in the season (September) suggesting good availability of grazing in October. Consumption of the LMFL increased from October-December. During this period the average daily temperatures were similar ranging from 8.7^{o}C in October to 7.2^{o}C in December, however due to short day length the opportunity for plant growth would have been limited reflected by reduced average estimated sward height and growth in this period. This is perhaps indicative of ewes increasing their consumption of the LMFL when grazing is limiting in availability due to seasonal changes. Similar observation was made by Aubel *et al.* (2011) who observed the consumption of a molasses based mineral feed block by cattle grazing in a rangeland environment to decrease when plants went from a state of dormancy to growth with season. The consumption of the block decreased in January which was the coldest month with little sward growth and sward height, this was thought to be due to winter silage being offered to ewes this month and their attention and preference for silage being greater than the LMFL. Consumption of the LMFL in February returned to a similar level of intake that was observed in December, at this stage ewes would be in the final trimester of pregnancy before lambing therefore

consumption of the LMFL was expected to be high due to foetal exponential growth. However, consumption of the LMFL decreased in March, this may have been a result of ewes preparing to lamb. Cabiddu *et al.* (2014) observed dairy ewes to have greater consumption of LMFL when the ADF content of forage was high suggesting ewes to consume more of the LMLF when grazing was perhaps nutritionally limiting. In this study wet chemistry was not conducted on sward samples to determine if any seasonal variation in the water-soluble carbohydrate, fibre (NDF, ADF, ADL) or crude protein content of grazing occurred, however dry matter was investigated. The dry matter content of sward samples from October to January were similar and of similar value to reference temperate grasses published by Ewing (2016). This was surprising as it was expected that the dry matter content of sward samples would increase during the winter months which was the case in February. Further research should be conducted to observe if any correlations could be made from LMFL consumption and the nutritional quality of forage.

3.6.2 Ewe Performance

Ewe performance was measured by recording the live weight of ewes and the scanning percentage of each group. Due to differences in the starting weights of the control and treatment groups, data were analysed on a percentage basis of the individual ewes starting live weight to see if LMFL supplementation had any effect on maintaining live weight. Data were analysed in two sections tupping-parturition (October-March) and post-partum-weaning (May-August) to account for weight loss post-partum as a result of the foetus. As expected, the live weight of ewes increased throughout the gestation period as a result of foetal growth. The group supplemented with the LMFL displayed greater live weight gain in comparison with the control group. Previous study by Cabiddu *et al.* (2014) demonstrated LMFL supplementation to have positive effects on the condition of dairy ewes pre and post-partum in the Mediterranean, in which ewes had better body condition scores and blood metabolic status in comparison to ewes in the control group. Likewise, studies in cattle have also demonstrated LMFL supplementation to have an increasing effect on live weight. Hart & Newbold (2015) demonstrated heifers extensively grazing in the autumn and spring in a temperate climate to have greater gains in live weight when supplemented with a LMFL. Likewise, Titgemeyer *et al.* (2004) demonstrated heifers consuming a diet of either Prairie hay or Prairie hay plus 1.96 g/ day of Alfalfa hay supplemented with a high protein (CP, 275 g/ kg DM) LMFL, to have greater daily live weight gains in comparison with heifers consuming the control diet.

It was expected that a difference between the live weights of ewes bearing twins and single ewes would occur pre-partum as a result of foetal weight. This was observed for the treatment group.

However, twin bearing ewes from the control group exhibited less weight gain pre-partum in comparison with single bearing ewes. This may be indicative of the demand for rearing twin lambs being metabolically higher and grazing alone being nutritionally inadequate to achieve this.

Ewe live weight declined post-partum as a result of foetal birth, with the group supplemented with the LMFL still displaying greater live weigh gain in comparison with the control group. During lactation (May-August) the live weight of ewes was expected to decrease due to the high metabolic demand of lactation, however this was not the case in either group or for single or twin bearing ewes. Sward samples were not measured during this period, however it would have been interesting to determine if this was due to the availability and nutritional quality of grazing being adequate for lactation.

It is well documented that nutrition can have a significant impact on the reproductive performance of animals in terms of viability, fecundity, prolificacy, physiological health and abortion (Ashworth *et al.*, 2009). In the control group eight ewes aborted with the majority of ewes aborting within the last trimester, whereas there were no ewes in the treatment group that aborted possibly suggesting the additional nutrition provided by the LMFL to be adequate for production. Likewise, scanning data revealed the group supplemented with LMFL to have a greater scanning percentage in comparison to the control group as a result of a greater number of ewes bearing twins. This may be indicative of the additional nutrition provided by the LMFL resulting in improved reproductive performance in terms of ovulation rate. Likewise, the duration of lambing in the control group was 49 days whilst the duration of lambing in the supplemented group was 22 days which is possibly indicative of ewes supplemented with LMFL conceiving within the first oestrous cycle when put to tup. This is of great benefice to the producer in that it reduces labour requirement and therefore cost of additional staff. However, it must be remembered that different rams were introduced to each group with a second ram joining each group 14 days after the first. Therefore, the duration of lambing may be associated with the ram fertility as well as ewe fertility. Therefore, further research is required with increased control measures put in place to determine if LMFL supplementation has any positive effects on the reproductive performance of ewes. However, previous research associated with the supplementary feeding of feed blocks to grazing ruminants in tropical, semi-arid or arid environments has demonstrated supplementation to have positive effects on reproductive performance. This is in terms of successful conception, parity and fecundity thought to be attributed to the additional nutrition the block provides to the diet (Salem & Nefzaoui, 2003) in which the formulations are at the discretion of the producer (Blache *et al.*, 2008). Likewise feed blocks have been demonstrated to improve the reproductive performance of rams, Anindo *et al.* (1998) demonstrated rams grazing tropical forages

of low nutritional value supplemented with molasses based urea feed blocks to have better semen quality and greater testicular growth in comparison with control rams.

3.6.3 Lamb Performance and Carcass Characteristics

It was not possible to record the birth weights of lambs as mentioned in section 3.6. Likewise, it was not possible record individual lamb daily live weight gains due to a lack of lamb identification as mentioned in section 3.6. However, the live weights of lambs within each group were recorded each month. Lambs who were supplemented with the LMFL on average had greater live weights each month in comparison with the control group. However, this does not take into account gender. Likewise, these results may be a result of lambs in the supplemented group being born earlier in the lambing period. Therefore, further research with increased control measures are required to fully determine the effect of LMFL supplementation on lamb birth weight and daily live weights gain.

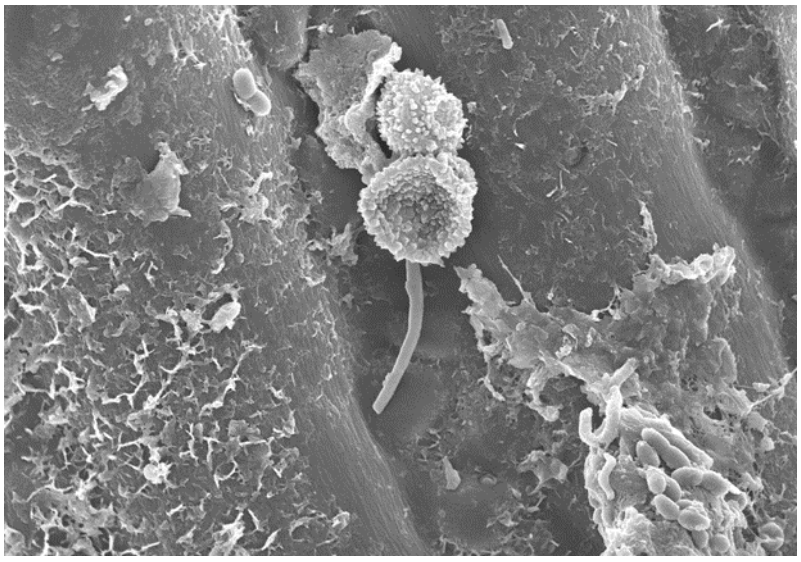
There have been no previous studies to investigate the effect of LMFL supplementation on carcass characteristics or grade awarded to lambs. Lambs were sent for slaughter when they were perceived as commercially viable by the farm resulting in inconsistencies in live weights at finishing and difficulties in comparing carcass grades. To make true comparisons between carcasses, lambs should have either been sent off for slaughter within x many days from birth or when they had reached a target live weight, for example 40 kg. Likewise, lambs were entered into two carcass specifications 1) small carcass and 2) large carcass with 11 out of 41 lambs in the control group being entered into the small carcass specification. This may be resultant of the nutritional quality and availability of grazing becoming limited for finishing due to the onset of winter and a reduction in plant growth, however this is based on anecdotal evidence. In addition, the lambing period was longer for the control group therefore this result may have been due to lambs in this category being the younger lambs of the group. As a result, lambs in the LMFL exhibited greater live weights before slaughter and thus greater carcass cold weights in comparison with the control group. However, there was no difference in the killing out percentage between groups. It was not possible to make sufficient comparisons between the carcass grades of the two groups due to carcasses being different weights and ages at slaughter. However, in both groups carcass grading was ideal for optimum payment. Therefore, further research is required to fully determine the effect of LMFL supplementation on the performance and carcass quality and characteristics of lambs.

3.7 Conclusion

In conclusion, the results of this trial were limiting due to experimental design and not enough control measures implemented and therefore should only be considered as preliminary results. However, this

small-scale study demonstrated the utilisation of LMFLs by grazing ewes to vary across the grazing season possibly in accordance with climate and limited availability of grazing. However, further research should be carried out to conclude this. LMFL supplementation appeared to have a positive effect on the live weight of ewes which agrees with the initial hypothesis made. Likewise, ewes demonstrated signs of improved reproductive performance in which a greater number of lambs were scanned *in-utero*. Likewise, the lambing period was shorter in comparison with the control group suggesting successful conception post ram turn out with added benefits of reduction in labour requirement for the producer. Lambs supplemented with the LMFL appeared to reach slaughter sooner in comparison with lambs in the control group. However, it was not conclusive if this was a result of lambs being born earlier in the lambing period, lambs having heavier weights at birth or if lambs had greater daily live weight gains. Overall, these results are indicative of the potential for LMFL supplementation to improve the performance of breeding ewes and their lambs in the upland. However, an up-scaled version of this study is required with review of experimental design and increased control measures before any absolute conclusions can be made.

Chapter 4 The Effect of Low Moisture Feed Lick Supplementation on Rumen Function and Microbiota *in-vitro*



4.1 Introduction

Ruminants are obligate herbivores with a unique digestive tract capable of utilising plant material regarded as human inedible (McDonald *et al.*, 2010). The rumen has been described as "the most elegant and highly evolved cellulose-digesting system in nature" (Weimer *et al.*, 2009) and is host to a highly diverse consortium of microbiota capable of degrading and fermenting plant material into products of utility to both the ruminant host and microbiota (Belanche *et al.*, 2019).

The nutritional quality and availability of grazing varies across the grazing season and at times is insufficient to meet the demands for production, especially for animals of high genetic merit or at physiologically demanding times such as growth, pregnancy and lactation. Beever *et al.* (1986) demonstrated variation in the nutritional composition of forages Perennial Ryegrass and White Clover cut early (May-June), mid (July) and late (August-September) in the grazing season, with the early and mid-cuts having a greater water-soluble carbohydrate (WSC) and lower nitrogen content in comparison with the late cut. Likewise, Cammell *et al.* (1986) demonstrated the dry matter, nitrogen and gross energy composition of Perennial Ryegrass and White Clover to differ between mid (May-June) and late (August-September) cuts with forages to be of a greater nutritional quality in the later cut. To compensate the provision of supplementary nutrition to the diet is common practice.

Low moisture feed licks (LMFL) are retailed as forage balancers, providing additional energy, protein, vitamins and minerals to the diet which may otherwise be limiting in forage. Previous research in cattle has demonstrated LMFL supplementation to have a complementary effect on the diet. In such studies, cattle consuming poor-quality tropical forages supplemented with LMFLs had increased voluntary forage intakes and increased digestibility of the diet in comparison with control animals (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Leupp *et al.*, 2005). Likewise, animals supplemented with LMFLs have exhibited improvements in animal performance in terms of live weight gain (Hart & Newbold, 2015) and improvements in body condition score and blood metabolic status (Cabiddu *et al.*, 2014). However, the biological basis of which LMFL supplementation effects the rumen is unknown. Therefore, using the *in-vitro* gas production technique this chapter aims to investigate the effect of LMFL supplementation on the rumen and aspects of the rumen microbiome.

4.2 Chapter Aims

Feed supplements can have differential effects on rumen metabolism such as complementation and addition. Complementation involves the supplement interacting with feed increasing its nutritional value, resulting in increases in feed intake and/ or digestibility. Addition describes the supplement as providing additional energy, protein, vitamin or mineral resources to the diet which act as a substrate for microbial metabolism (Carro *et al.*, 2005). It is hypothesised in this thesis that LMFL

supplementation has a stimulating effect on rumen microbiota, thereby increasing the degradation and fermentation potential of forage. In addition, Chapter 3 demonstrated LMFL supplementation to have a positive effect on the performance of breeding ewes in the uplands, therefore a study involving the *in-vitro* gas production technique will be conducted to investigate the effect of the LMFL supplement on the rumen and aspects of the rumen microbiome in the aim of explaining outcomes on animal performance.

This chapter consists of three experiments in which Experiments 1 and 2 are preliminary experiments in preparation for the main experiment of this chapter, Experiment 3. Through these experiments this chapter will focus on the effect of the LMFL on the degradation of forage, rumen fermentation and microbial biomass in the absence and presence of forage. This chapter will also focus on the gas production technique with the aim of developing a sufficient protocol for future studies in this thesis.

Experiment 1: The effect of different doses of low moisture feed lick supplementation on the temporal degradation of forage, rumen fermentation and bacterial biomass

Experiment 2: The effect of different doses of low moisture feed lick supplementation on rumen fermentation and microbial biomass

Experiment 3: The effect of low moisture feed lick supplementation on rumen fermentation and the microbial biomass and activity of the potential metabolically active solid associated population

4.2.1 Hypothesis

H_1: LMFL supplementation will increase the dry matter degradation of forage

H_2: LMFL supplementation will increase rumen fermentation whereby the volume of gas produced and the molar concentrations of volatile fatty acids and ammonia will increase

H_3: LMFL supplementation will have an increasing effect on microbial biomass of the solid and liquid associated populations

H_4: LMFL supplementation will have an increasing effect on the activity of microbial enzymes associated with carbohydrate metabolism

4.3 Experimental Design

All experiments had ethical approval as described in Chapter 2 section 2.1. The experimental designs for all experiments were based on an *in-vitro* colonisation study by Mayorga *et al.* (2016) as described in Chapter 2 section 2.2. The dietary substrate used in all experiments was Ryegrass hay and the LMFL supplement was Crystalyx® Extra High Energy as described in Chapter 2 section 2.2.1. The LMFL supplement was formulated into the diet based on the ratio of a 70 kg ewe consuming 2% of its live weight in forage dry matter (1.4 kg DM/ day) and 50 g of LMFL, which is a reported typical daily intake of the LMFL in commercial use (personal communication Crystalyx® UK). Diets were scaled down to achieve the same ratio of diet DM to vessel size as previously conducted by Mayorga *et al.* (2016). Chemical analysis of the Ryegrass hay and the LMFL was carried out as described in Chapter 2 section 2.6 to determine the; dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), crude protein (CP), ether extract (EE) and ash content (Table 4.1). The NDF and ADF content of the LMFL was not determined due to the trace quantity of fibre within the product.

Table 4.1: Chemical analysis of dietary substrates Ryegrass hay and the low moisture feed lick

	Ryegrass Hay	[a]LMFL
Dry Matter (g/ kg)	859	964
Organic Matter (g/ kg DM)	928	875
Neutral Detergent Fibre (g/ kg DM)	654	ND
Acid Detergent Fibre (g/ kg DM)	347	ND
Crude Protein (g/ kg DM)	58.5	121
Ether Extract (g/ kg DM)	12.5	110
Ash (g/kg DM)	71.9	125

[a]LMFL = Low moisture feed lick Crystalyx® Extra High Energy (Caltech-Crystalyx UK, Siloth, UK)
ND = Not detected

4.3.1 Experiment 1

This experiment is a preliminary experiment to establish suitable methodology in preparation for the main experiment of this chapter (Experiment 3).

To 40 ml Wheaton bottles 0.28 g (0.24g DM) of fresh chopped hay (1-2 cm) was added along with the LMFL where appropriate at levels x0 (0 mg), x1 (8.65 mg), x2 (17.3 mg) and x10 (86.5 mg) the original formulated dose. Control bottles containing the supplement in the absence of forage were included in experimental design along with a blank control of no forage or supplement.

Rumen fluid was collected from one sheep as described in Chapter 2 section 2.2.2, combined with a phosphate bicarbonate buffer to make a 1:9 v/v inoculant as described in Chapter 2 section 2.2.3 and 24 ml dispensed into bottles and stored in an upright incubator set at 39^{o}C as described in Chapter 2 section 2.2.4. There were three biological replicates of each treatment and seven different incubation times of 0.5, 1, 2, 4, 6, 8, and 24 hours, in which there were 24 bottles per time point totalling 168 bottles.

Before the sacrificial harvest of bottles the volume of gas that had accumulated in the head space of the bottles was recorded as described in Chapter 2 section 2.2.5. Bottles were harvested using a porcelain Buchner funnel as described in Chapter 2 section 2.2.7. A sub-sample of the liquid fraction was recovered the pH recorded and relevant sub-samples recovered for measuring the molar concentrations of volatile fatty acids (VFAs) within samples as described in Chapter 2 sections 2.5.1 and 2.5.2 respectively. A further sub sample of the liquid fraction was recovered, immediately flash frozen in liquid-N and freeze dried for 48 hours. The residual forage was wrapped in pre-weighed tin foil, immediately flash frozen in liquid-N and freeze dried for 48 hours. The dry matter degradation of the forage was calculated as described in Chapter 2 section 2.6.2.

DNA was extracted from the residual liquid and solid freeze dried samples from the 4 and 24 hour time points as described in Chapter 2 section 2.9.1 and quantified via spectrophotometry as described in Chapter 2 section 2.9.3. The relative abundance of bacterial microbiota of the liquid (LAP) and solid (SAP) associated populations was conducted using the quantitative polymerase chain reaction technique (qPCR) relative to an environmental standard as described in Chapter 2 section 2.9. Time points 4 and 24 hours were specifically chosen to target the colonisation of substrate by microbiota early and late on in the colonisation process. Previous studies have identified microbial colonisation to be biphasic involving colonisation by a primary and secondary colonising community (Huws *et al.*, 2016). Previous research investigating the colonisation of fresh Perennial Ryegrass by rumen microbiota demonstrated the events of primary colonisation to last for 2-4 hours before shifting to secondary colonisation events post 4 hours (Huws *et al.*, 2013, Huws *et al.*, 2016, Mayorga *et al.*, 2016). However the events of colonisation have been demonstrated to vary in time with different forages due to differences in forage biochemistries, physiologies (Huws *et al.*, 2014) and genotypes (Kingston-Smith *et al.*, 2013). Elliott *et al.* (2018) demonstrated the events of primary colonisation of fresh Birds Foot and fresh Red Clover to take up to 6 hours and the primary colonisation events of fresh Perennial Ryegrass to take up to 4 hours before shiftting to secondary colonisation events. The conservation of forage has also been demonstrated to effect the events of colonisation, Belanche *et al.* (2017) demonstrated the primary colonisation of fresh Perennial Ryegrass hay to take up to 3 times

as long in compariosn with fresh Perennial Ryegrass. Therefore to ensure the primary and secondary colonising community were targeted time points 4 and 24 hours were selected.

4.3.2 Experiment 2

This experiment is another preliminary experiment to establish methodology in preparation for the main experiment of this chapter, Experiment 3. The experimental design of this experiment has been modified based on the findings from Experiment 1. In Experiment 1 it was found that 168 bottles was a large sample volume to work with and at times resulted in technical difficulty in harvesting bottles. It was also found that measuring gas pressure before sacrificial harvest resulted in inconsistencies in the gas curve, therefore it was decided that the repeated measure of accumulated gas would be a more practical method of measuring gas volume as it would allow for the calculation of gas volume cumulatively.

Experimental design was the same as described in section 4.3.1 where 0.28 g (0.24g DM) of chopped hay was weighed in to 40 ml Wheaton bottles. Doses of the LMFL supplement consisted of doses, x0, x1 and x10, in which the x1 dose consisted of 8.65 mg of the LMFL formulated into the diet as discussed in section 4.3. Control bottles containing the LMFL in the absence of substrate were included in experimental design. Bottles were inoculated with 24 ml of inoculant in which the rumen fluid was collected from one sheep and diluted 1:9 v/v with a phosphate bicarbonate buffer as described in section 4.3.1. There were three biological replicates of each treatment totalling 18 bottles.

The cumulative volume of gas was measured from the head space of all bottles at time points, 1, 2, 4, 6, 8 and 24 hours as described in section 4.3.1. The kinetics of fermentation were calculated as described in Chapter 2 section 2.2.6 to determine the maximal potential for fermentation and the rate of fermentation based on gas production data. All bottles underwent sacrificial harvest at 24 hours where the liquid and solid fractions were separated via a tea strainer as described in section 4.3.1. A sub-sample of the liquid fraction was recovered for measuring pH and the molar concentrations of VFAs as described in section 4.3.1. A further sub-sample of the liquid fraction and the entirety of the solid fraction was immediately flash frozen in liquid-N and freeze dried for 48 hours in preparation for molecular work. Freeze dried residual liquid and solid samples were processed to quantify the relative abundance of bacteria, methanogens and anaerobic fungi associated with the LAP and SAP using the q-PCR technique as described in section 4.3.1.

4.3.3 Experiment 3

This is the main experiment of this chapter with experimental design upscaled and modified based on findings from Experiments 1 and 2. It was found that LMFL supplementation had no effect on the

microbial biomass of the total (DNA) LAP or SAP population. Therefore, in this experiment microbiota from the potential metabolically active population (RNA) were targeted.

To 250 ml Duran bottles (Sigma-Aldrich, Missouri, USA) 1.7 g (1.5g DM) of chopped hay was added along with 108 mg of the LMFL (x2 dose). Control bottles containing no forage for each treatment were included in experimental design. Rumen fluid was collected from four sheep as described in section 4.3.1 to increase biological replication and four different inoculants were made. Inoculants consisted of rumen fluid and a phosphate bicarbonate buffer in a ratio of 1:9 v/v as described in section 4.3.1. Bottles were inoculated with 150 ml of the inoculant and placed in an upright standing incubator set at 39^{o}C for the duration of the experiment. There were three biological replicates of each treatment and two time points for sacrificial harvest, 4 and 24 hours totalling 96 bottles.

The cumulative volume of gas was measured from the head space of all bottles at time points, 1, 2, 4, 6, 8 and 24 hours and the kinetics of fermentation calculated as described in section 4.3.2. Half of all bottles underwent sacrificial harvest at 4 hours and the remaining half at 24 hours. The liquid and solid fractions were separated by pouring bottle contents through a tea strainer as described in section 4.3.2. A sub-sample of the liquid fraction was recovered for measuring pH and the molar concentrations of VFAs as described in section 4.3.1. The entirety of the solid fraction recovered was immediately flash frozen in liquid-N and stored at -80^{o}C for later molecular work.

RNA was extracted from a sub-sample of the residual solid sample as described in Chapter 2 section 2.9.2 and quantified via spectrophotometry as described in Chapter 2 section 2.9.3. RNA was reversed transcribed into complementary DNA (cDNA) as described in Chapter 2 section 2.9.4 and the relative abundance of bacteria, methanogens, anaerobic fungi and protozoa of the SAP quantified using the q-PCR technique as described in section 4.3.1.

A further sub-sample of the residual solid sample was recovered, freeze dried for 48 hours and processed to determine the activity of microbial enzymes associated with carbohydrate metabolism as described in Chapter 2 section 2.7. Enzymes were extracted from samples using a phosphate buffer as described in Chapter 2 section 2.7.1. The activity of amylase, carboxymethyl-cellulase (CMCase) and xylanase was measured using substrates, starch and carboxymethyl-cellulose and xylan (Chapter 2 section 2.7.2) via a series of reducing sugar assays as described in Chapter 2 section 2.7.3. The content of protein within samples was determined via the Bradford assay as described in Chapter 2 section 2.7.4 to determine enzymatic activity reactive to the protein content of samples.

4.4 Statistics

All statistical analysis was conducted using Genstat 19th edition and statistical significance was accepted as $p < 0.05$.

4.4.1 Experiment 1

Statistical analysis of the gas data was conducted via repeated measures analysis of variance (ANOVA). Data regarding fermentation parameters (pH, VFAs) and microbial biomass was measured via two-way ANOVA, in which factors consisted of the supplement and time.

4.4.2 Experiment 2

Statistical analysis of the gas data was conducted as described in section 4.4.1. Statistical analysis of fermentation parameters (pH, VFAs), the kinetics of gas production and microbial biomass was analysed via one-way ANOVA.

4.4.3 Experiment 3

Statistical analysis of the gas data was conducted as described in section 4.3.1. Statistical analysis of fermentation kinetics was conducted via one-way ANOVA with blocking for sheep. Statistical analysis of fermentation parameters (pH, VFAs, ammonia), microbial biomass and enzymatic activity were measured via two-way ANOVA with the supplement and time as factors and blocking for sheep.

4.5 Results

The *in-vitro* gas production technique was used to measure the effect of LMFL supplementation on the rumen. This technique is a popular method for evaluating the degradation of feeds and rumen fermentation within a closed system (Ørskov & McDonald, 1979, Theodorou *et al.*, 1994). The technique can also be used to determine safe doses of dietary additives, supplements and secondary compounds and for investigations into the rumen microbial population (de la Fuente *et al.*, 2017). The technique is relatively simple, non-time consuming and inexpensive to run in comparison with *in-vivo* studies (Carro *et al.*, 2005).

4.5.1 Experiment 1

The effect of different doses of the LMFL supplement on the degradation potential of forage was measured. LMFL supplementation had no effect on the dry matter degradation of forage, however there was a trend for dry matter degradation to increase in the presence of the supplement (Table 4.2). The degradation of forage significantly increased with time ($p=0.018$). There were no interactions of supplementation with time.

The effect of different doses of the LMFL in the absence and presence of forage on rumen fermentation was measured. Data are presented separately for supplementation in the absence and presence of forage due to the presence of forage being an additional source of substrate for microbial fermentation resulting in greater fermentation parameters. Traditionally in gas production experiments control bottles containing the inoculum in the absence of dietary substrate would be subtracted from treatment samples to account for any endogenous/ soluble substrates present in the rumen fluid (Carro *et al.*, 2005). Although control bottles were run in this experiment, they were not subtracted from treatment samples. This was to gain an insight into the effect of the supplement alone on rumen fermentation.

In the absence of forage LMFL supplementation had the effect of increasing the volume of gas produced ($p<0.001$) (Table 4.2), with gas production being sustained for up to 8 hours (Figure 4.1). Moreover, supplementation of the diet at the x2 dose and above was significantly greater in comparison to the control treatment ($p<0.001$) (Table 4.2). Dosage at the x10 level resulted in a significantly greater volume of gas being produced which was of a similar volume to hay on its own. In the presence of forage gas production was sustained over 24 hours (Figure 4.1). LMFL supplementation at all doses had the effect of increasing the volume of gas produced, with supplementation at the x10 dose having the greatest effect. ($p<0.001$) (Table 4.2). Likewise, the extent of gas production was greater in comparison with the supplement in the absence of forage. In both the absence ($p<0.001$) and presence ($p<0.001$) of forage the volume of gas produced increased significantly with time. There was a significant interaction of the LMFL supplement with time ($p<0.001$).

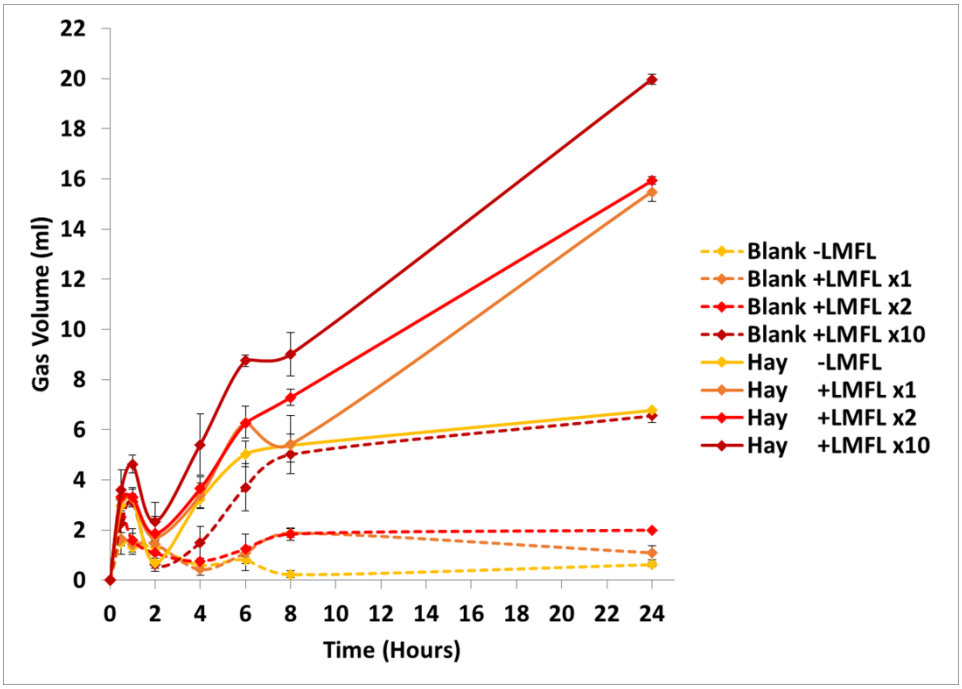


Figure 4.1: Effect of different doses (x1, x2 and x10) of low moisture feed lick supplementation (LMFL) in the absence (---,Blank) and presence (-,Hay) of forage on the volume of gas generated at time intervals 0.5, 1, 2, 4, 6, 8 and 24 hours of incubation in rumen fluid. Data points (◆) represent the mean total gas volume readings of the three biological replicates and error bars are the standard error of the mean.

Fermentation parameters, inoculum pH and the molar concentrations of the VFAs were measured in the absence and presence of forage to gain information about the potential pathways of fermentation and the results are presented in Table 4.2. In the absence of forage LMFL supplementation had the effect of reducing the pH of the inoculum when the LMFL was provided at the x2 dose and above ($p=0.006$). However, pH did not deviate below optimal conditions for fermentation most likely because of the phosphate bicarbonate buffer in the inoculum. A similar observation was made in the presence of forage where the LMFL supplement had the effect of reducing the pH of the inoculum ($p=0.039$), however this was when the x10 dose was provided. The pH of the inoculum decreased with time both in the absence ($p=0.020$) and presence ($p<0.001$) of forage. There was an interaction between the supplement and time in the absence of forage ($p=0.037$), however this was not observed in the presence of forage.

The molar concentration of total VFAs and individual molar proportions of the VFAs were measured to give an indication of the potential pathways of fermentation (Table 4.2). In the absence of forage

LMFL supplementation at the x2 dose and above had an increasing effect on the molar concentration of total VFAs ($p<0.001$), which was a similar observation to that made in the presence of forage ($p=0.004$) but to a greater extent. Time had the effect of increasing the molar concentration of the total VFAs both in the absence ($p=0.018$) and presence ($p<0.001$) of forage. There was a significant interaction of supplementation and time in the absence of forage ($p=0.007$) and a tendency for an interaction in the presence of forage ($p=0.051$).

In the absence of forage LMFL supplementation had the effect of increasing effect the molar concentration of the major VFAs; acetate ($p<0.001$) and propionate at the x10 dose ($p<0.001$) and butyrate at the x2 dose and above ($p<0.001$). Likewise, LMFL supplementation in the presence of forage had the effect of increasing the molar concentrations of acetate ($p=0.001$), propionate ($p=0.003$) and butyrate ($p=0.010$) at the x2 dose and above. LMFL supplementation had no effect on the molar concentration of the branched chain VFAs in the absence or presence of forage. In the absence of forage time had the effect of increasing the molar concentrations of acetate ($p=0.049$), propionate ($p=0.02$), butyrate ($p=0.012$), the branched chain VFAs ($p<0.001$). Similar observation was made in the presence of forage where the molar concentrations of acetate, propionate, butyrate and the branched chain VFAs all increased with time ($p<0.001$). In the absence of substrate significant interactions were observed between supplementation and time for the VFAs, acetate ($p=0.018$), propionate ($p=0.003$) and butyrate ($p=0.002$). In the presence of substrate significant interactions were observed between supplementation and time for the VFAs propionate ($p=0.002$) and butyrate ($p=0.022$).

The proportion of acetate to propionate (A:P) was measured to indicate the potential pathway of fermentation as described in Chapter 2 section 2.5.2. In the absence of substrate LMFL supplementation had the effect of decreasing A:P when supplemented at the x2 dose and above ($p<0.001$). However, in the presence of forage LMFL supplementation had the effect of increasing A:P at the x10 dose ($p<0.001$). In the absence of substrate time had the effect of decreasing A:P ($p<0.001$) which was a similar observation made in the presence of substrate ($p<0.001$). There was a significant interaction between supplementation and time for A:P both in the absence ($p<0.001$) and presence of forage ($p<0.001$).

Microbial biomass from the kingdom bacteria was measured relative to an environmental sample using the q-PCR technique (Table 4.3). In the absence and presence of forage LMFL supplementation had no effect on the relative abundance of bacteria of the LAP. Likewise, time had no effect on the relative abundance of bacterial biomass and there were no interactions between the supplement and time. LMFL supplementation had no effect on the relative abundance of bacteria from the SAP. Time

had no effect on the relative abundance of bacteria from the SAP and there was no interaction between the supplement and time.

Table 4.2: Effect of different doses of the low moisture feed lick on fermentation parameters in the absence and presence of forage over 24 hours

	-LMFL	+LMFL x1	+LMFL x2	+LMFL x10	sed	Supplement	Time	Supplement x Time
Absence of forage								
Gas volume (ml)	0.91[a]	1.31[a]	1.57[b]	3.23[c]	0.162	<0.001	<0.001	<0.001
pH	7.31[a]	7.26[ab]	7.18[bc]	7.12[c]	0.095	0.006	0.020	0.037
Total volatile fatty acids	2.17[a]	2.34[ab]	2.63[b]	3.33[c]	0.145	<0.001	0.018	0.007
Acetate (mM)	1.20[a]	1.25[a]	1.39[a]	1.71[b]	0.069	<0.001	0.049	0.018
Propionate (mM)	0.42[a]	0.47[ab]	0.58[a]	0.87[c]	0.026	<0.001	0.020	0.003
Butyrate (mM)	0.32[a]	0.34[ab]	0.38[b]	0.47[c]	0.021	<0.001	0.012	0.002
Branched chain (mM)	0.23	0.27	0.27	0.27	0.039	0.660	<0.001	0.113
A:P	2.74[a]	2.64[b]	2.43[c]	2.07[d]	0.079	<0.001	<0.001	<0.001
Presence of forage								
Dry matter degradation (%)	12.6	17.9	17.5	16.1	0.610	0.487	0.018	0.540
Gas volume (ml)	3.95[a]	5.54[b]	5.94[b]	7.63[c]	0.284	<0.001	<0.001	<0.001
pH	7.11[a]	7.02[a]	7.04[ab]	6.89[b]	0.037	0.039	<0.001	0.082
Total volatile fatty acids	3.69[a]	4.21[ab]	4.61[b]	5.29[c]	0.294	0.004	<0.001	0.051
Acetate (mM)	1.90[a]	2.18[ab]	2.39[b]	2.80[c]	0.138	0.001	<0.001	0.079
Propionate (mM)	0.96[a]	1.12[ab]	1.22[b]	1.45[c]	0.087	0.003	<0.001	0.002
Butyrate (mM)	0.48[a]	0.55[ab]	0.60[bc]	0.66[c]	0.039	0.010	<0.001	0.022
Branched chain (mM)	0.35	0.33	0.34	0.43	0.031	0.081	<0.001	0.948
A:P	2.06[a]	2.06[a]	2.11[a]	2.33[b]	0.029	<0.001	<0.001	<0.001

[a,b,c,d] Means within row not bearing a common later differ

Table 4.3: Effect of different doses of the low moisture feed lick on the relative abundance of bacteria from the solid and liquid associated populations at 4 and 24 hours

Log copy/ g DM	-LMFL	+LMFL x1	+LMF x2	+LMFL x10	sed	4 Hours	24 Hours	sed	Supplement	Time	Supplement x Time
Absence of forage											
LAP	10.41	10.75	9.14	11.35	0.895	10.35	10.47	0.633	0.131	0.856	0.564
Presence of forage											
LAP	11.08	10.87	11.42	11.52	0.688	11.00	11.45	0.487	0.770	0.374	0.404
SAP	11.17	10.55	9.46	11.07	0.645	10.14	10.98	0.456	0.065	0.083	0.325

LAP = Liquid associated population

SAP = Solid associated population

4.5.2 Experiment 2

LMFL supplementation demonstrated the effect of increasing the volume of gas produced over 24 hours (Figure 4.2) in the absence (p=0.048) and presence (p=0.007) of forage at the x10 dose (Table 4.4). In the absence of forage there was little difference between the cumulative volume of gas generated by the control and x1 LMFL dose, which both reached an asymptote at 8 hours (Figure 4.2). However, when the x10 dose was provided gas production was sustained over 24 hours and demonstrated a similar if not greater effect on gas production in comparison with the forage treatment. Likewise, the kinetics of fermentation calculated using exponential model by Ørskov and McDonald, (1979) revealed the LMFL at the x10 dose to have an increasing effect on the maximal potential for fermentation (a+b) both in the absence (p=0.003) and presence (p<0.001) of forage. However, the supplement had no effect on the rate (c) of fermentation for both treatments (Table 4.4). Time had the effect of increasing the volume of gas produced over 24 hours both in the absence (p<0.001) and presence (p<0.001) of forage and there was a significant interaction of the supplement with time in both the absence (p<0.001) and presence (p=0.004) of forage.

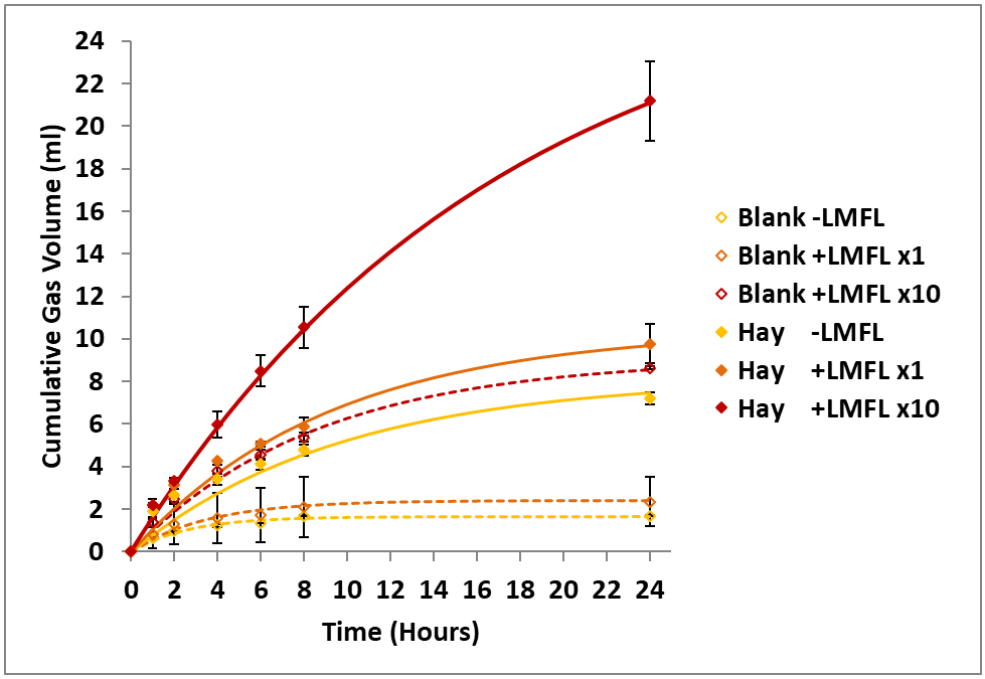


Figure 4.2: Effect of different doses (x1 and x10) of low moisture feed lick (LMFL) supplementation in the absence (Blank, - -) and presence (Hay, -) of forage on the cumulative volume of gas generated over 24 hours. Data points represent the mean cumulative gas volume readings of biological replications at time intervals 1, 2, 4, 6, 8 and 24 hours and error bars are the standard error of the mean. Lines represent the predicted cumulative volume of gas calculated from the exponential equation $Y= a + b (1 – e^{(-ct)})$ by Ørskov and McDonald, (1979).

Table 4.4: Effect of different doses of low moisture feed lick in the absence and presence of forage on gas production parameters over 24 hours

	-LMFL	+LMFL x1	+LMFL x10	sed	p-Value
Absence of forage					
[1]Cumulative gas volume (ml)	1.26[a]	1.65[a]	4.37[b]	0.761	0.048
a+b	1.65 [a]	2.42[a]	9.11[b]	0.670	0.003
c	0.38	0.29	0.12	0.146	0.320
Presence of forage					
[2]Cumulative gas volume (ml)	4.00[a]	5.05[a]	8.62[b]	0.539	0.007
a+b	8.13[a]	10.43[a]	28.02[b]	1.689	<0.001
c	0.10	0.11	0.06	0.032	0.333

a+b = Maximal potential of fermentation

c = Rate of fermentation

[a,b] Means within row not bearing a common later differ

[1]Time ($p<0.001$), supplement x time ($p<0.001$)

[2]Time ($p<0.001$), supplement x time ($p=0.004$)

Fermentation parameters; pH and the total molar concentration of the VFAs were measured at 24 hours which was the end point of the experiment (Table 4.5). In the absence and presence of forage LMFL supplementation had no effect on the pH of the inoculum. In the absence of forage LMFL supplementation at the x10 dose had the effect of increasing the total molar concentration of the total VFAs ($p<0.001$). Similar observation was made in the presence of forage but to a greater extent ($p=0.003$) as a result of the additional dietary substrate.

In the absence of forage LMFL supplementation at the x10 dose had the effect of increasing the molar concentrations of the major VFAs; acetate ($p<0.001$), propionate ($p<0.001$) and butyrate ($p<0.001$). Similar observation was made in the presence of forage where the molar concentration of acetate ($p=0.003$), propionate ($p=0.005$) and butyrate ($p=0.006$) increased with supplementation but to a greater extent. In comparison with treatments in the absence of forage. LMFL supplementation at the x10 dose demonstrated a reducing effect on the A:P in the absence ($p<0.001$) and presence of forage ($p=0.007$). There was no effect of the LMFL supplement on the molar concentration of the branched chain VFAs in the absence of forage. However, LMFL supplementation had an increasing effect on the molar concentration of the branched chain VFAs in the presence of forage when provided to the diet as the x10 dose.

The effect of LMFL supplementation on the relative abundance of microbial biomass from the microbial kingdoms; bacteria, methanogens and anaerobic fungi were measured via q-PCR (Table 4.6). In the absence of forage LMFL supplementation had no effect on the relative abundance of bacteria or anaerobic fungi of the LAP. However, there was a tendency for LMFL supplementation to have the effect of increasing the relative abundance of methanogens ($p=0.060$), however this observation was not made in the presence of forage or in the SAP. In the presence of forage LMFL supplementation had no effect on the relative abundance of bacteria and methanogens of the LAP. However LMFL supplementation at the x10 dose had the effect of reducing the microbial biomass of anaerobic fungi of the LAP ($p=0.032$). LMFL supplementation had no effect on the relative abundance of bacteria, methanogens or anaerobic fungi of the SAP.

Table 4.5: Effect of different doses of low moisture feed lick in the absence and presence of forage on fermentation parameters at 24 hours

	-LMFL	+LMFL x1	+LMFL x10	sed	p-Value
Absence of forage					
pH	6.98	6.98	6.85	0.066	0.154
Total volatile fatty acids (mM)	2.11[a]	2.44[a]	4.76[b]	0.142	<0.001
Acetate (mM)	1.00 [a]	1.20[a]	2.40[b]	0.107	<0.001
Propionate (mM)	0.30 [a]	0.43[a]	1.20[b]	0.042	<0.001
Butyrate (mM)	0.24[a]	0.29[a]	0.57[b]	0.107	<0.001
Branched chain (mM)	0.26	0.28	0.30	0.025	0.835
A:P	3.29[c]	2.77[b]	2.00[a]	0.090	<0.001
Presence of forage					
pH	6.98	7.01	7.07	0.090	0.625
Total volatile fatty acids (mM)	6.23[a]	6.04[a]	9.03[b]	0.565	0.003
Acetate (mM)	3.31[a]	3.09[a]	4.64[b]	0.301	0.004
Propionate (mM)	1.56[a]	1.53[a]	3.05[b]	0.323	0.005
Butyrate (mM)	0.69[a]	0.66[a]	0.81[b]	0.031	0.006
Branched chain (mM)	0.34[b]	0.37[b]	0.27[a]	0.027	0.019
A:P	2.12[a]	2.01[a]	1.55[b]	0.120	0.007

[a,b] Means within row not bearing a common later differ

Table 4.6: Effect of different doses of low moisture feed lick on microbiota of the liquid and solid associated populations in the absence and presence of forage at 24 hours

Log copy/ g DM	-LMFL	+LMFL x1	+LMFL x10	sed	p-Value
Absence of forage					
LAP: Bacteria	11.43	11.38	11.44	0.084	0.786
LAP: Methanogens	9.18	9.47	9.41	0.100	0.060
LAP: Anaerobic Fungi	6.63	6.69	6.63	0.109	0.092
Presence of forage					
LAP: Bacteria	11.52	11.52	11.61	0.122	0.659
LAP: Methanogens	9.48	9.54	9.35	0.102	0.224
LAP: Anaerobic Fungi	7.63^{b}	7.66^{b}	7.06^{a}	0.188	0.032
SAP: Bacteria	11.52	11.42	11.13	0.241	0.320
SAP: Methanogens	9.10	9.11	9.31	0.089	0.103
SAP: Anaerobic Fungi	8.61	8.74	8.25	0.230	0.162

^{a,b} Means within row not bearing a common later differ

LAP = Liquid associated population

SAP = Solid associated population

4.5.3 Experiment 3

Experiment 3 is the main experiment of this chapter with experimental design based on findings and experiences from Experiments 1 and 2.

The cumulative volume of gas was measured over 24 hours (Figure 4.3). In the absence of forage gas production was sustained over 8 hours before reaching an asymptote, however in the presence of forage gas production was sustained over 24 hours. In the absence of forage LMFL supplementation had no effect on the cumulative volume of gas produced over 24 hours. However in the presence of forage LMFL supplementation had the effect of increasing the volume of gas generated over 24 hours ($p=0.034$) (Table 4.7). The kinetics of fermentation were calculated using the exponential model by Ørskov and McDonald, (1979) and revealed LMFL supplementation to have no effect on the maximal potential for fermentation (a+b) or the rate of fermentation in the absence of forage. Likewise, in the presence of forage LMFL supplementation had no effect on the maximal potential for fermentation (a+b) which was surprising regarding the LMFL demonstrating the effect of increasing the volume of gas produced over 24 hours. Similarly, there was no effect of LMFL supplementation on the rate of fermentation. Time had the effect of increasing the volume of gas produced over 24 hours both the absence ($p=0.002$) and presence ($p<0.001$) of forage. There were no interactions between the supplement and time in the absence or presence of forage.

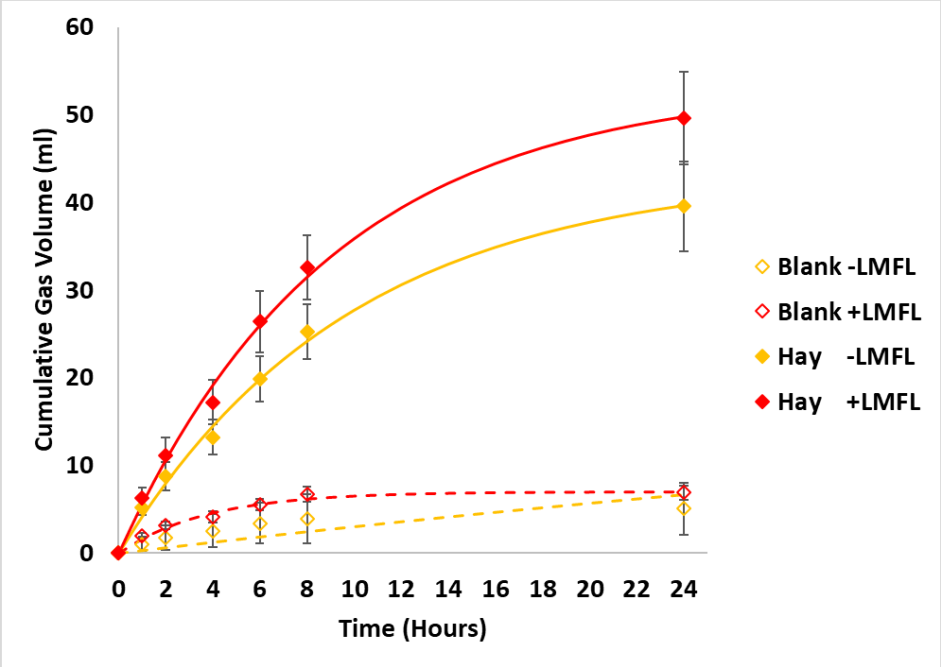


Figure 4.3: Effect of low moisture feed lick (LMFL) supplementation in the absence (Blank, - -) and presence (Hay, -) of forage on the cumulative volume of gas generated over 24 hours. Data points (◆) represent the mean cumulative gas volume readings for the four biological replicates taken at time intervals 1, 2, 4, 6, 8 and 24 hours and error bars are the standard error of the mean. Lines represent the predicted cumulative gas curves calculated from the exponential equation $Y= a + b (1 – e^{(-ct)})$ by Ørskov and McDonald, (1979).

Table 4.7: Effect of low moisture feed lick supplementation in the absence and presence of forage on the cumulative volume of gas and the kinetics of fermentation over 24 hours

Fermentation Parameter	-LMFL	+LMFL	sed	p-Value
Absence of forage				
[1]Cumulative gas volume (ml)	3.69	4.73	1.478	0.556
a+b	8.10	7.80	2.640	0.904
c	0.27	0.27	0.078	0.986
Presence of forage				
[2]Cumulative gas volume (ml)	18.6	23.9	1.418	0.034
a+b	52.8	58.5	8.430	0.552
c	0.11	0.12	0.023	0.613

- Absence of the LMFL
+ Presence of the LMFL
a+b = Maximal potential for fermentation
c = Rate of fermentation
[1]Time (P=0.002), Supplement x Time (P=0.737)
[2]Time (P<0.001), Supplement x Time (P=0.331)

The effect of LMFL supplementation on fermentation parameters; inoculum pH and the molar concertation of VFAs and ammonia was measured (Table 4.8). Supplementation had no effect on the pH of the inoculum in the absence or presence of forage. Time had no effect on the pH of the inoculum in the absence of forage, however in the presence of forage the pH of the inoculum at 4 hours was lower in comparison with the pH at 24 hours ($p<0.001$). There were no significant interactions of the supplement with time in the absence or presence of forage.

LMFL supplementation had the effect of increasing the total molar concentration of the total VFAs in the absence ($p=0.003$) and presence ($p=0.031$) of forage, with the molar concentration of VFAs being greatest in the presence of forage due to additional substrate for fermentation by rumen microbiota. Time had the effect of increasing the molar concentration of VFAs both in the absence ($p=0.001$) and presence ($p<0.001$) of forage. There were no significant interactions between the supplement and time.

In the absence of forage LMFL supplementation had the effect of increasing the molar concentration of the major VFAs; acetate ($p<0.001$) and propionate ($p<0.001$), however no effect on the molar concentration of butyrate was observed. Supplementation had no effect on the molar concentration of the branched chain VFAs. In the presence of forage LMFL supplementation had the effect of increasing the molar concentrations of acetate ($p=0.003$), however no effect of LMFL supplementation was observed on the molar concentrations of propionate, butyrate or the branched chain VFAs. LMFL supplementation had a reducing effect on the ratio of A:P in the absence of forage ($p<0.001$), however no effect on the ratio was observed in the presence of forage. Time had the effect of increasing the molar concentration of propionate ($p=0.002$), butyrate ($p=0.020$) and the A:P ($p=0.003$) and decreasing the molar concentration of the branched chain VFAs ($p=0.001$) in the absence of forage. Likewise, time had the effect of increasing the molar concentration of acetate ($p<0.001$), propionate ($p<0.001$), butyrate ($p=0.020$) and the branched chain VFAs ($p<0.001$). However, time also demonstrated a reducing effect on A:P ($p=0.017$). No significant interactions between the supplement and time were detected in the absence or presence of forage.

LMFL supplementation in the absence and presence of forage had no effect on the molar concentration of ammonia. In the absence ($p=0.035$) of forage time had the effect of decreasing the molar concentration of ammonia and there was a tendency for a similar effect in the presence of forage ($p=0.080$). There were no interactions between the supplement with time in the absence or presence of forage.

Table 4.8: Effect of low moisture feed lick (LMFL) supplementation on fermentation parameters at 4 and 24 hours

	-LMFL	+LMFL	sed	4	24	sed	Supplement	Time	Supplement x Time
Absence of forage									
pH	7.06	7.04	0.032	7.02	7.08	0.029	0.428	0.085	0.354
Total VFAs (mM)	0.84	1.00	0.066	0.83	1.01	0.040	0.003	0.001	0.738
Acetate (mM)	0.38	0.45	0.012	0.41	0.41	0.012	<0.001	0.868	0.348
Propionate (mM)	0.13	0.18	0.007	0.16	0.14	0.004	<0.001	0.002	0.043
Butyrate (mM)	0.20	0.23	0.038	0.25	0.17	0.030	0.354	0.020	0.820
Branched Chain (mM)	0.14	0.15	0.029	0.19	0.11	0.017	0.639	0.001	0.962
A:P	3.01	2.55	0.126	2.61	2.95	0.082	<0.001	0.003	0.915
Ammonia (mM)	7.04	7.19	1.025	8.19	6.05	0.864	0.865	0.035	0.578
Presence of forage									
pH	7.02	6.99	0.051	7.09	6.92	0.017	0.080	<0.001	0.867
Total VFAs (mM)	1.71	1.87	0.349	1.22	2.36	0.063	0.031	<0.001	0.066
Acetate (mM)	0.84	0.90	0.161	0.60	1.13	0.017	0.003	<0.001	0.040
Propionate (mM)	0.41	0.46	0.107	0.26	0.61	0.021	0.075	<0.001	0.146
Butyrate (mM)	0.26	0.29	0.037	0.24	0.32	0.041	0.366	0.020	0.379
Branched Chain (mM)	0.20	0.22	0.060	0.12	0.29	0.014	0.470	<0.001	0.339
A:P	2.13	2.12	0.221	2.38	1.87	0.174	0.962	0.017	0.770
Ammonia (mM)	6.15	5.60	0.700	6.50	5.25	0.628	0.410	0.080	0.409

- Absence of the LMFL
+ Presence of the LMFL

The relative abundance of bacteria, methanogens, anaerobic fungi and protozoa of the potential metabolically active SAP were measured via reverse transcription q-PCR (Table 4.9). LMFL supplementation had no effect on the relative abundance of bacteria, methanogens, anaerobic fungi or protozoa. Time had the effect of increasing the relative abundance of bacteria ($p<0.001$) and anaerobic fungi ($p=0.001$) and there was a tendency for the relative abundance of protozoa to increase with time ($p=0.069$). There were no significant interactions between the supplement and time.

To better understand the effect of the LMFL on the metabolism of carbohydrates by rumen microbiota the potential activity of the SAP was indirectly measured from their enzymatic activity via a series of reducing sugar assays (Table 4.10). LMFL supplementation had no effect on the potential activity of enzymes; amylase, CMCase or xylanase. Likewise, time had no effect on the activity of amylase, CMCase or xylanase. There were no interactions of the supplement with time. The protein content within samples was measured via the Bradford assay to obtain an indirect picture of the potential enzyme population. LMFL supplementation had no effect on the protein content within samples. However, time had the effect of increasing the protein content within samples ($p=0.033$). There were no interactions of the supplement with time. The potential activity of enzymes was calculated relative to the protein content within samples. There was no effect of LMFL supplementation on the potential activity of amylase, CMCase or xylanase. Likewise, time had no effect on the activity of amylase, CMCase or xylanase. There were no significant interactions of the supplement and time.

Table 4.9: Effect of low moisture feed lick (LMFL) supplementation on the relative abundance of microbiota from the potentially active sold associated population at 4 and 24 hours

Log copy/ g DM	-LMFL	+LMFL	sed	4	24	sed	Supplement	Time	Supplement x Time
Bacteria	9.02	8.99	0.345	8.51	9.50	0.1726	0.869	<0.001	0.872
Methanogens	7.77	7.83	0.115	7.70	7.90	0.101	0.602	0.110	0.993
Anaerobic Fungi	6.49	6.58	0.280	6.14	6.93	0.152	0.577	0.001	0.880
Protozoa	6.51	6.70	0.280	6.36	6.85	0.245	0.423	0.069	0.199

- Absence of the LMFL

+ Presence of the LMFL

Table 4.10: Effect of low moisture feed lick supplementation on the enzymatic activity of the solid associated microbial population at 4 and 24 hours

	-LMFL	+LMFL	sed	4	24	sed	Supplement	Time	Supplement x Time
Absolute enzyme activity (mMol sugar/ g DM / minute)									
Amylase	0.310	0.308	0.006	0.306	0.312	0.006	0.767	0.305	0.600
Carboxymethyl-cellulase	0.029	0.033	0.003	0.030	0.033	0.003	0.129	0.307	0.029
Xylanase	0.147	0.151	0.011	0.143	0.155	0.011	0.700	0.301	0.491
Relative enzyme activity (mMol sugar/ g protein / minute)									
Protein (mg/ g DM)	6.18	6.09	0.203	5.92	6.35	0.175	0.627	0.033	0.993
Amylase	0.825	0.823	0.038	0.822	0.827	0.034	0.958	0.890	0.993
Carboxymethyl-cellulase	0.078	0.090	0.007	0.087	0.087	0.009	0.133	0.393	0.350
Xylanase	0.390	0.405	0.038	0.405	0.405	0.036	0.712	0.489	0.502

- Absence of the LMFL
+ Presence of the LMFL

4.6 Discussion

The aim of this chapter was to investigate the effect of LMFL supplementation on rumen function and rumen microbiota. To do this, investigations were made into the effect of LMFL supplementation on; the degradation of forage within the rumen, the effect of the supplement on rumen fermentation and the effect of the supplement on microbial biomass of the LAP and SAP in the absence and presence of forage. This chapter also looked at the development of the *in-vitro* gas production technique to determine a suitable method for future experiments in this thesis.

4.6.1 Degradation of forage in the Rumen

Ryegrass hay was selected as a substrate in all *in-vitro* experiments. This particular forage was selected as it is one of the most popular forages grown for grazing ruminants in the UK (Kingston-Smith *et al.*, 2013). Hay was selected rather than fresh grass due to hay having a poorer nutritional quality in terms of a higher dry matter content and lower energy, sugar and protein content in comparison with fresh grass. It was therefore hypothesised that an effect of the LMFL would likely be seen using a poorer quality forage as the LMFL is specifically designed to balance forage based diets. Likewise, previous studies investigating the effect of LMFL supplementation on the metabolism of cattle were fed diets of conserved low quality tropical forages such as; Prairie hay, Brome hay, Alfalfa hay and Switch grass hay (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Titgemeyer *et al.*, 2004, Leupp *et al.*, 2005).

Fresh hay chopped to 1-2 cm was used instead of dried milled substrate as is traditional in gas production experiments. This was because certain aspects of this chapter aimed to investigate the colonisation of dietary substrate by rumen microbiota. Likewise, it was felt if the substrate was dried and ground into a fine homogenous powder it would not be representative of the physical structure of the substrate available in the rumen and could possibly impact fermentation by increasing the accessibility of the substrate for microbial degradation and fermentation, thereby potentially biasing the result. Moreover the use of fresh substrate is in coherence with published *in-vitro* studies investigating the temporal colonisation of forage by rumen microbiota (Huws *et al.*, 2013, Huws *et al.*, 2014, Huws *et al.*, 2016, Mayorga *et al.*, 2016, Belanche *et al.*, 2017, Elliott *et al.*, 2018). However, it must be remembered that inclusion of fresh substrate is not entirely representative of how the forage would be presented to the rumen as it does not consider damage to plant tissues as a result of mastication.

The dry matter degradation of forage was measured in Experiment 1 and demonstrated the LMFL supplement to have no effect on the percentage of forage degraded over 24 hours. However, there was a trend for the LMFL to have an increasing effect on the percentage of forage degraded in comparison with the control treatment. Reasons for supplementation statistically having no effect on the degradation of forage may be associated with difficulties encountered when harvesting the bottles. It was found that handling small quantities of dietary substrate increased the likelihood of technical error occurring and variation between sample replicates. Samples were initially harvested by separating the liquid and solid fractions via vacuum filtration using a porcelain Buchner funnel, however this resulted in forage getting stuck in the pores of the funnel resulting in recovery loss. Alternative methods of sample recovery were tested including vacuum filtration using glass crucibles fitted with sintered filters, straining bottle contents through muslin and straining bottle contents through a tea strainer. It was found the tea strainer was the most effective method of sample recovery due to having finer sieve in comparison with the Buchner funnel and filtering through glass crucibles was time consuming and made the separation of the solid and liquid fractions more time consuming. Therefore, the forage degradation results from this Chapter should be reviewed with caution. Furthermore, the percentage of forage that had degraded over the 24 hours was low in comparison with what was expected, however this may have been a result of the concentration of the inoculum being weak (1:9.v/v). Further investigations into the effect of LMFL supplementation on the degradation of forage within the rumen using the gas production technique are made in Chapter 5 and investigations *in-sacco* are made in Chapters 5 and 6.

There are limited studies in the literature investigating the effect of LMFL supplementation on rumen function *in-vitro*. However, the observation made in Experiment 1 is in support of an *in-vitro* study by Chaudhry (2008), who observed a low (120 g/ kg DM) and high (250 g/ kg DM) protein LMFL supplement to have an increasing effect on the dry matter degradation of grass nuts and barley straw over 72 hours of incubation in buffered rumen fluid obtained from slaughter cattle. However, Chaudhry (2008) observed no difference between the percentage of forage degraded for the low or high protein LMFL and there was no difference in the percentage of forage degraded when the dose (90 vs 180 g / kg forage) was doubled. In Experiments 1 and 2 different doses of the LMFL were investigated to determine the best dose to use in the main experiment of this chapter (Experiment 3). In Experiment 1 LMFL supplementation at the x1 and x2 dose resulted in a similar percentage of dry matter being degraded, however supplementation at the x10 dose displayed a lower percentage of forage dry matter being degraded in comparison with the other doses. This is perhaps indicative of the x10 dose being too high a dose for optimum rumen function, possibly due to high sugar content creating a less favourable environment for rumen fiber digesting microbiota. Likewise, in Experiment

2 the microbial biomass of anaerobic fungi decreased at the x10 dose suggesting this dose to have a negative effect on this microbial kingdom. Moreover, anaerobic fungi play an important role within the microbiome in which they have the ability to produce and secrete enzymes capable of degrading lingo-cellulolytic plant material as described in Chapter 1 section 1.3.4. As a result, the x2 dose was selected as the dosage for the final experiment to ensure no negative effect would occur on rumen function. The average DMD of forage across samples in experiment 1 was lower in comparison to those observed by Chaudhry (2008), this is possibly due to the concentration of inoculum in this study being weaker (1:9 v/v) in comparison (1:4 v/v).

Studies investigating the effect of LMFL supplementation on the degradation of forage *in-vitro* are limited. However, studies have been conducted in whole animal trials in cattle. These studies demonstrated juvenile cattle consuming poor quality tropical forages supplemented with various LMFLs to have a complementary effect on the diet, in which the digestibility of the diet is enhanced and in turn voluntary forage intakes are greater in comparison with control animals. Greenwood *et al.* (1998) demonstrated beef steers fed Prairie grass hay *ad-libitum* supplemented with a LMFL to increase voluntary forage OM intake by 13%, NDF intake by 12%, N intake by 10% and increase N digestibility of the diet by 56% in comaprison with control animals. Similar observation was made in a follow up study by Greenwood *et al.* (2000) who demonstrated steers fed Prairie grass hay *ad-libitum* supplemented with LMFLs of varying base ingreedients (cane molasses, beet molasses and a concetrated seperator by-product from the sugar industry) to increase forage OM, NDF and N intakes on average by 22% and to increase the OM digestibility of the diet by 8% and the N digestibility of the diet by 61% in comparison with the control diet. Löest *et al.* (2001) observed a tendency for LMFLs varying in non-protein nitorgen sources (urea vs urea plus biuret) to increase voluntary forage DM, OM, NDF and CP intakes by 22% and the digestibility of dietary DM and OM by 18%, dietary NDF by 19% and dietary CP by 57% on avearage in comaparison with the control diet. Leupp *et al.* (2005) observed a tendency for steers fed Switch grass hay *ad-libitum* supplemented with LMFLs containing various test dietary additives (fermentation products, seaweed) to have the effect of increasing voluntary forage OM intake by 6% , CP intake by 5% and have a tendency to increase the digestibility of dietary OM by 35% and CP by 30%. However these observatons are in disagreement with Titgemeyer *et al.* (2004) who observed steers fed diets of Alfalfa hay, Brome hay or a mix of Brome and Alfalfa hay supplemented with LMFLs to have no effect on voluntary forage DM, OM, NDF or CP intakes or the digestibility of DM, OM or NDF. However, diets supplemented with the LMFL demonstrated an increasing effect on the digestibility of dietary N by 4.3%, however this is most likely attributed to the additional protein provided to the diet by the LMFL. In an additional study by Titgemeyer *et al.* (2004), heifers consuming diets of Praire hay *ad-libitum* or a mixture of Praire hay

ad-libitum plus 1.96 kg/ day of Alfalfa hay supplemented with either a low (144 g CP/ kg DM) or high (275 g CP/ kg DM) protein LMFL had no effect on voluntary forage DM intake. However, there was a trend for heifers supplemented with the low protein LMFL to have reduced forage intakes in comaprison with the control and high protein LMFL. Moreover, LMFL supplementation had the greatest effect on the poorer quality forage diet of Praire hay where the DM digestibility of the diet increased on average by 13%, however the supplement had no effect on the digestibility of the better quality diet of Prarire hay when supplemnted with Alfalfa hay. These results are possibly indicative of LMFL supplementation having the greatest effect on the degradation and digestibility of forage when forage is of a low nutritional quality and limiting in nutrition, thus highlighting its use as a forage balancer.

4.6.2 Rumen Fermentation

Rumen microbiota are responsible for degrading and fermenting dietary substrate into products of utility to the ruminant host and microbiota itself (Edwards *et al.*, 2017). Previous research in juvenile cattle has demonstrated LMFL supplementation to have a complementary effect on the diet whereby it has been observed to have stimulating effects on the digestibility of the diet and voluntary forage intakes as discussed in section 4.6.1. However, there is little research into the effect of the LMFL supplement on rumen fermentation, especially in sheep. The LMFL used in this study was Crystalyx® Extra High Energy (Caltech-Crystalyx, Siloth, UK) as described in Chapter 2 section 2.2.1. This LMFL has a high sugar content (300 g/ kg DM) as a result of the base ingredient being cane molasses. Cane molasses are a by-product from the sugar refining industry and renowned for having a high sugar content (650 g/ kg DM sugar) in particular sucrose, which provides a readily available source of fermentable metabolisable energy to the diet (Ewing, 2016). Simple carbohydrates such as sugars are rapidly fermented within the rumen by microbiota (Sniffen *et al.*, 1992) into the VFAs and gases (carbon dioxide, hydrogen, methane) are produced as a by-product of this process. Therefore, it was hypothesised that the supplement would have an increasing effect on fermentation within the rumen.

The effect of the LMFL on rumen fermentation was measured in both the absence and presence of forage to better understand the effect the supplement alone has on the rumen. Moreover, different doses of the LMFL were investigated (Experiments 1 and 2) to determine the best dose to use in the main experiment of this chapter (Experiment 3). The x2 dose was selected as the x10 dose appeared to have a reducing effect on the degradation of forage and on the biomass of anaerobic fungi (section 4.6.1). Moreover, the x1 dose was based on the typical daily intake of the LMFL by a breeding ewe as described in Chapter 2 section 2.2.1, therefore it was felt that supplementing more than double the daily dose would not be representative or a realistic dose.

In the absence of forage LMFL supplementation at the x2 dose and above had an increasing effect on the volume of gas produced. Moreover, when the x10 dose was provided the volume of gas produced was a similar volume to that produced by hay itself (Experiments 1 and 2). The kinetics of fermentation demonstrated LMFL supplementation at the x10 dose to have an increasing effect on the maximal potential for fermentation (a+b), however there was no effect on the rate of fermentation (c) (Experiment 2). A similar observation was not made for gas production or the kinetics of gas production in the main experiment of this chapter which involved supplementation at the x2 dose (Experiment 3). Gas production in all experiments was sustained for up to 8 hours before reaching an asymptote (Experiments 1, 2 and 3). An asymptote was most likely reached early on in fermentation due to dietary substrate for fermentation being limited. In the presence of forage LMFL supplementation at the x2 and x10 dose had an increasing effect on the volume of gas generated over 24 hours (Experiments 1, 2 and 3). Gas production was sustained over the 24 hours and the volume of gas produced greater in comparison with treatments in the absence of forage. The kinetics of fermentation demonstrated LMFL supplementation at the x10 dose to increase the maximal potential for fermentation (a+b), however there was no effect on the rate of fermentation (Experiment 2). A similar observation was not made for the kinetics of gas production at the x2 dose in the main experiment of this chapter (Experiment 3). These results are perhaps indicative of the LMFL enhancing fermentation within the rumen. Moreover they are indicative of the LMFL being used as a substrate for microbial fermentation within the rumen.

The gas data in Experiment 1 should be viewed with caution and viewed only as preliminary data. In Experiment 1 the volume of gas that had accumulated in the head space of vessels was measured directly before sacrificial harvest at time points 0.5, 1, 2, 4, 6, 8 and 24 hours. As a result, the gas curve generated was not smooth (Figure 4.1) and thus effected the ability to calculate the kinetics of fermentation via modelling. Recording the volume of gas that had accumulated directly before harvest was a limitation in the experimental design. For example, López *et al.* (2007) emphasises the importance of regular venting of gas from bottles suggesting when the head space becomes saturated this can have a negative effect on microbial activity and thus impede fermentation. Theodorou *et al.* (1994) demonstrated pressure in the head space of bottles exceeding 7 psi to negatively affect microbial fermentation. Moreover, Theodorou *et al.* (1994) stressed that when the head space of bottles were saturated any further gases produced were likely to dissolve in the inoculum as a result of Henry's law, thus creating an unfavourable environment for certain microbiota as well as underestimating total gas production. The pressure readings in Experiment 1 exceeded 7 psi at time intervals 8 and 24 hours, therefore adjustments to the experimental design were made. For subsequent experiments (Experiments 2 and 3) the cumulative volume of gas was measured with

regular venting of bottles post pressure reading. This new adaptation to the protocol also allowed for sample size to be reduced as a result of the repeated measure of gas. In Experiment 1 it was also found that 168 bottles was too high a sample number and increased the likelihood of technical error occurring. In addition, biological repetition could not be increased by using rumen fluid from more than one sheep due to the scale of bottles that would be required.

The pH of the inoculum was measured in response to fermentation, where the production of VFAs and lactate have the effect of reducing rumen pH due to their acidic nature. In the absence of forage LMFL supplementation at the x2 dose and above had the effect of reducing the pH of the inoculum (Experiment 1). Likewise, in the presence of forage LMFL supplementation at the x10 dose had the effect of reducing the pH of the inoculum (Experiment 1). However, these results were not reciprocated in the other experiments (Experiments 2 and 3) and pH did not fall below the optimum pH for fermentation. It was expected that little change would occur to the pH as a response of the phosphate bicarbonate buffer in the inoculum, which sequesters H^+ ions and generates carbon dioxide thus helping to maintain pH. Therefore, these results are somewhat limited. However, the effect of LMFL supplementation on rumen pH is investigated *in-vivo* in Chapter 6.

The diet is known to influence the molar concentrations of VFAs and can be used as an intervention for manipulating the pathways of fermentation for production and environmental benefice (Davis, 1967). In the absence of forage LMFL supplementation at the x2 dose and above had the effect of increasing the molar concentration of total VFAs (Experiment 1, 2 and 3). A similar observation was made in the presence of forage but to a greater extent as a result of the additional dietary substrate (Experiments 1, 2 and 3). These results are indicative of the LMFL acting as a substrate for fermentation by rumen microbiota. These results disagree with those made *in-vivo* by Greenwood *et al.* (2000) who observed steers fed Prairie grass hay *ad-libitum* supplemented with a cane molasses based LMFL to have no effect on the molar concentration of total VFAs. However, when steers were supplemented with either a beet molasses or concentrated separator by-product based LMFL the molar concentration of total VFAs increased. However, Leupp *et al.* (2005) observed steers consuming Prairie grass hay *ad-libitum* supplemented with a beet molasses based LMFL to have no effect on the molar concentration of total VFAs. It must be remembered that LMFL are a type of feed supplement and there are different products with different dietary ingredients, inclusion rates of ingredients and specifications thereby making comparisons between studies difficult. Therefore, the effects of the LMFL on rumen fermentation are likely to be attributed to block specification. In addition, it must be remembered that the *in-vitro* gas production system is a closed system and doesn't take into account certain events of rumen metabolism such as; rumen retention time, rumen flow rate and the absorption of the products of fermentation therefore comparisons between *in-vitro* and *in-vivo* trials

should be viewed with caution. In this chapter the molar concentration of total VFAs varied between experiments with Experiment 3 having the lowest molar concentration in comparison with the other experiments. This was surprising as Experiment 3 was an up-scaled version of Experiments 1 and 2. Likewise the molar concentration of VFAs was generally low. This is most likely associated with the inoculum being weak with a 1:9 v/v ratio of rumen fluid to buffer.

The molar concentration of individual VFAs were measured to give an indication of the potential pathways of fermentation. In the absence of forage LMFL supplementation at the x2 dose appeared to have an increasing effect on the molar concentration of acetate and propionate (Experiment 3). Similar observations were made at the x10 dose in which the molar concertation of acetate, propionate and butyrate increased (Experiments 1 and 2). Supplementation had no effect on the molar concentration of the branched chain VFAs. In the presence of forage LMFL supplementation at the x2 dose and above had an increasing effect on the molar concentration of acetate, propionate and butyrate (Experiments 1, 2 and 3). There was no effect of LMFL supplementation on the branched chain VFAs (Experiment 1 and 3) aside from at the x10 dose in which the concentration decreased (Experiment 2). Overall, these results are indicative of the potential for LMFL supplementation to have an enhancing effect on fermentation in the absence and presence of forage. This is most likely attributed to the LMFL being utilised by rumen microbiota as a substrate for fermentation and is most likely why fermentation was further enhanced when the dose was increased in this chapter.

The observations made in this chapter differ to those made *in-vivo* in cattle. Greenwood *et al.* (2000) observed steers consuming Prairie grass hay *ad-libitum* supplemented with a cane molasses based LMFL to have no effect on the individual proportions of acetate, propionate or butyrate, however LMFL supplementation had the effect of reducing the molar concentration of iso-butyrate and iso-valerate (branched chain VFAs). In addition, when diets were supplemented with a beet molasses based and a concentrated separator by-product based LMFL, both supplements had the effect of increasing the molar proportions of acetate, propionate and butyrate and the effect of decreasing the molar proportions of iso-butyrate, iso-valerate and valerate (branched chain VFAs). There was also a trend for the concentration of acetate to decrease at the expense of butyrate over time for all LMFL tested. Likewise, Leupp *et al.* (2005) saw no effect of a beet molasses based LMFL on the individual molar concentrations of acetate, propionate or butyrate when fed to steers consuming a diet of Switch grass hay. Collectively the results of this chapter and studies in cattle are indicative of different LMFLs having different effects on rumen fermentation. This is most likely attributed to the LMFLs having different specifications and containing different dietary ingredients resulting in variation in the energy, protein, vitamin and mineral contents between licks and therefore influencing the extent of fermentation.

The ratio of A:P was measured to gain a further understanding of the potential pathway of fermentation as described in Chapter 2 section 2.5.2. Increases in the ratio of A:P are indicative of increased acetate production whilst a reduction in A:P is indicative of increased propionate production. In the absence of forage the LMFL had the effect of decreasing A:P at the x1 dose and above (Experiment 1 and 2 and 3). In the presence of forage LMFL supplementation had the effect of increasing the A:P at the x10 dose (Experiment 1), however this was not reciprocated throughout this chapter in which the A:P was also observed to decrease at the x10 dose (Experiment 2) and have no effect at all (Experiment 3). Increased molar concentrations of propionate have been associated with the inclusion of rapidly fermentable carbohydrates to the diet such as starch and water-soluble carbohydrates (WSC). Penner *et al.* (2009) demonstrated the inclusion of starch at increasing levels to the diets of Holstein cattle to increase the molar concentration of total VFAs and propionate. Davis (1967) observed the molar concentration of acetate to decrease when lactating dairy cows were fed high starch low fibre diets. Lee *et al.* (2002) observed an increase in the molar proportion of propionate at the expense of acetate when steers were fed different varieties of Perennial Ryegrass of high WSC contents. Similar observation was made by Lee *et al.* (2003) *in-vitro* using the rumen simulated technique (RuSiTec). However, Taweel *et al.* (2005) observed no difference in the total or individual molar concentrations of VFAs when lactating dairy cows were fed varieties of Perennial Ryegrass of low and high WSC contents. The LMFL used in this chapter had a sugar content of 300 g/ kg DM sugar, therefore it was hypothesised that the added sugar in the diet could induce a similar result to the studies described above. However, in this chapter propionate did not decrease at the expense of acetate or any other VFAs measured. The delivery of the LMFL in this chapter was a flat rate dose, therefore only providing a small quantity of additional dietary substrate to be fermented by rumen microbiota and may be why such observations were not made.

The molar concentration of ammonia was measured as an insight into protein metabolism within the rumen. The LMFL in this study had a CP content of 120 g CP/ kg DM of which 5% was protein equivalent of urea. It was hypothesised that an increase in the molar concentration of ammonia may arise from the de-amination of rumen degradable protein to ammonia. However, in the absence and presence of forage LMFL supplementation had no effect on the molar concentration of ammonia (Experiment 3). This observation disagrees with Greenwood *et al.* (2000) and Leupp *et al.* (2005) whom both observed LMFL supplementation to have the effect of increasing the molar concentration of ammonia in the rumen. However, the LMFLs fed in these studies had more than double the protein content in comparison to the LMFL fed in this chapter.

4.6.3 Microbial Biomass

Rumen microbiota are responsible for the degradation and fermentation of dietary substrate into products of utility for the host. The fermentation of plant structural carbohydrates into VFAs by rumen microbiota generates ATP which can be utilised by microorganisms for growth (Kingston-Smith *et al.*, 2008). However, (Hackmann & Firkins, 2015) emphasises that not all ATP is directed towards microbial growth but instead some is utilised for maintenance, the synthesis of reserve carbohydrates and energy spilling.

It was hypothesised that LMFL supplementation would result in increased microbial growth due to the additional energy and protein the LMFL provides to the diet. Initially microbiota from the LAP and SAP associated microbial populations were investigated (Experiments 1 and 2), however for the main experiment (Experiment 3) only the SAP was investigated. This was decided as the SAP is renowned for being the largest sub-population of microbiota within the rumen and associated with the degradation of plant structural carbohydrates into products for fermentation (McAllister *et al.*, 1994). Moreover, previous research in cattle has demonstrated the potential for LMFL supplementation to increase the degradation of forage *in-vitro* (Chaudhry, 2008) and the digestibility of poor quality forages *in-vivo* (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Leupp *et al.*, 2005), therefore it was hypothesised that this sub-population would be the main population driving this effect.

DNA was extracted from residual fluid and solid samples and the relative abundance of microbiota in the total microbial population determined via q-PCR (Experiments 1 and 2). In the absence of forage LMFL supplementation had no effect on the relative abundance of bacteria or anaerobic fungi of the total LAP (Experiments 1 and 2). However, a tendency for LMFL to have the effect of increasing the relative abundance of methanogens in the LAP was observed (Experiment 2). No previous research has been conducted on the effect of LMFL supplementation on the methanogenic community, likewise the volume of methane was not measured in this study. However, study by Hart & Newbold (2015) demonstrated grazing heifers supplemented with LMFLs to have no effect on enteric methane emissions.

In the presence of forage LMFL supplementation had no effect on the relative abundance of bacteria or methanogens of the total LAP (Experiments 1 and 2), however the relative abundance of anaerobic fungi decreased at the x10 dose (Experiments 2). This is perhaps indicative of the x10 dose having a negative effect on the anaerobic fungal population. Moreover, anaerobic fungi are heavily involved with the degradation of fibrous plant material (Edwards *et al.*, 2008) and the forage used as a substrate in this chapter was hay which has a high fibre content, therefore a decrease in the fungal community

was not expected. Belanche *et al.* (2012) demonstrated cattle fed high starch diets to have a reducing effect on the concentration of anaerobic fungi present in rumen fluid, however the concentration of methanogens and protozoa also decreased which was an observation not made in the is experiment. LMFL supplementation had no effect on the relative abundance of bacteria, methanogens or anaerobic fungi of the total SAP. It was therefore concluded that the LMFL must be having a stimulating effect on microbial activity. Therefore, the effect of LMFL supplementation on the potential metabolically active SAP was measured following the extraction of RNA from residual solid samples (Experiment 3). In this experiment LMFL supplementation had no effect on the relative abundance of bacteria, methanogens, anaerobic fungi or protozoa of the potential metabolically active SAP. Although there was no effect of LMFL supplementation on the relative abundance of microbial biomass that doesn't mean to say that there is no effect of LMFL supplementation on the microbial community structure and the diversity of microbiota within the different kingdoms. Therefore further research is required to determine if LMFL supplementation has an effect on the structure and diversity of microbial populations.

The relative abundance of bacteria of the total LAP and SAP was measured at 4 and 24 hours (Experiment 1). Time had no effect on the microbial abundance of bacteria of the total LAP in the absence or presence of forage, and there was little difference between the relative abundance of the bacteria in the absence or presence of forage. Likewise, time had no effect on the total bacterial SAP and there was little difference between the relative abundance of microbiota of the SAP or LAP. This was surprising as the *in-vitro* system is a closed system and it was thought time would have an increasing effect on microbial biomass as a result of microbial growth and the progressive events of microbial colonisation. Likewise, the SAP is the largest sub-population of microbiota within the rumen therefore it was expected that this population would be greater in abundance in comparison with the LAP. Time had the effect of increasing the relative abundance of bacteria and anaerobic fungi of the potential metabolically active SAP (Experiment 3). This agrees with an *in-vitro* study by Huws *et al.* (2014) who demonstrated the abundance of bacteria colonising the leaf and stem fractions of fresh Perennial Ryegrass to increase over 24 hours. A similar observation was made by Mayorga *et al.* (2016) who also observed time to have an increasing effect on the abundance of bacteria colonising fresh Perennial Ryegrass *in-vitro*. Likewise, Liu *et al.* (2016) demonstrated time to have an increasing effect on the abundance of bacteria colonising Rice straw and Alfalfa straw over 48 hours *in-sacco*. Using RuSiTec fermenters Belanche *et al.* (2017) demonstrated the colonisation of Perennial Ryegrass and Perennial Ryegrass hay by bacteria, methanogens and protozoa to be greater at 4 hours in comparison with 24 hours. However, Belanche *et al.* (2016) observed time to have no effect on the abundance of bacteria, methanogens, anaerobic fungi or protozoa of the LAP over 24 hours when RuSiTec

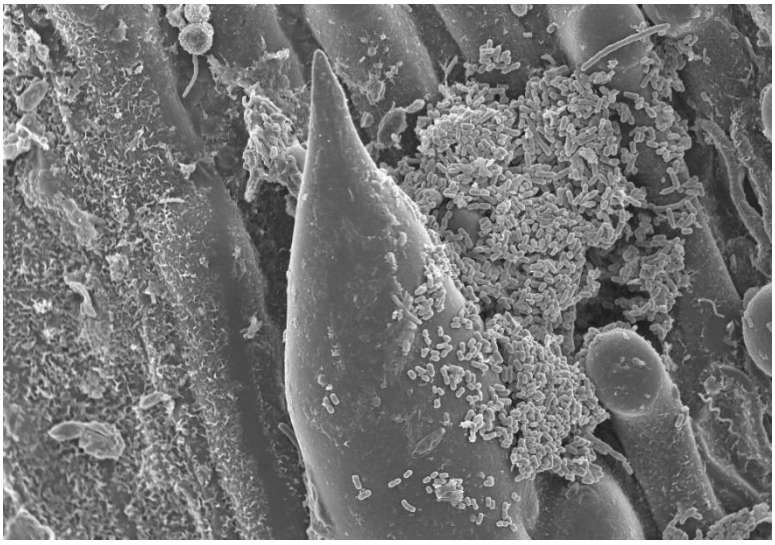
fermenters were used to investigate the supplementation of a forage concentrate (50:50) diet with different varieties of seaweed. These results are indicative of LMFL supplementation having no effect on the relative abundance of microbiota of the LAP or SAP. This suggests that microbiota do not utilise the LMFL as a substrate for microbial growth. Moreover, LMFL supplementation tended to increase the dry matter degradation of forage (section 4.6.1) and enhance rumen fermentation (section 4.6.2) this suggests the LMFL to have a stimulating effect on microbial metabolism.

The metabolic activity of the SAP associated with carbohydrate metabolism was measured indirectly through a series of reducing sugar assays (Experiment 3). LMFL supplementation had no effect on the activity of Amylase, CMCase or Xylanase. Likewise, time had no effect on the activity of enzymes. The protein content of samples was measured as an indirect indication of the enzyme content within samples. LMFL supplementation had no effect on the protein content within samples, however time had the effect of increasing the protein content of samples. Reasons why LMFL supplementation demonstrated no effect on microbial enzyme activity may be due to the forage being low in nutritional quality and having a finite resource of rapidly degradable carbohydrates. Likewise, the LMFL was supplied as a flat rate dose so was likely to be fermented rapidly on entry into the rumen. Moreover, the results in this study are lower in comparison with values obtained by Belanche *et al.* (2016) and Belanche *et al.* (2016) however the nutritional composition of dietary substrates in these studies were of a better nutritional value and the protein content detected in samples greater in comparison with this study.

4.7 Conclusion

In conclusion, LMFL supplementation demonstrated the potential to have both a complementary and additive effect on the diet *in-vitro*. LMFL supplementation demonstrated the potential to increase the degradation of forage within the rumen. In the absence and presence of forage LMFL had the effect of enhancing rumen fermentation suggesting microbiota to utilise the LMFL as a substrate for fermentation, most likely a result of the LMFL having a high sugar content. The LMFL demonstrated no effect on the relative abundance of microbiota of the LAP or SAP suggesting the LMFL to not induce microbial growth but instead stimulate microbial metabolism.

Chapter 5 Effect of Low Moisture Feed Lick Supplementation on Rumen Metabolism and the Solid Associated Bacterial Population



5.1 Introduction

The rumen microbiome consists of a plethora of highly diverse microbiota across several kingdoms (Stewart *et al.*, 2019) that work in symbiosis with the host and in synergy with one another to digest and ferment recalcitrant plant material into products of utility (volatile fatty acids, ammonia) for microbial and host survival (Piao *et al.*, 2014). Traditionally rumen microbiota were isolated from rumen samples, grown on media using culture techniques and identified via their morphology, shape and in the case of bacteria by Gram stain methods (Matthews *et al.*, 2019). However, not all rumen microbiota can be successfully cultured (Zehavi *et al.*, 2018), it is estimated as little as 15% of rumen bacteria have been successfully cultured (Morgavi *et al.*, 2013). This is a major limitation in microbiome studies, however molecular biology and bioinformatics analysis is proving a popular alternative technique with the capability of studying the complexity, diversity, potential function and potential activity of members of the microbiome with potential of identifying microbiota as far as genus level.

Next generation sequencing (NGS) is a popular culture independent molecular technique for the metataxonomic analysis of the microbiome. This in conjunction with the "omic" techniques such as metagenomics, metatranscriptomics, proteomics and metabolomics (Figure 5.1) provides additional information into the potential function and metabolic activity of microbiota within the rumen (Huws *et al.*, 2018). The use of DNA in NGS is popular due to DNA being robust and easy to handle in comparison with RNA which degrades easily (Tan & Yiap, 2009). However, Popova *et al.* (2010) argues that sequencing of DNA is not indicative of microbial metabolic activity, viability or growth highlighting the importance of studies using RNA. However Blazewicz *et al.* (2013) cautions the idea that RNA is not entirely representative of the "active" microbial population quoting "while all growing organisms are active, not all active organisms are growing" and explains how dormant microorganisms can contain high levels of ribosomes, therefore to assume RNA is directly correlated with microbial activity may be misleading and instead it would be more correct to refer to microbiota sequenced from RNA as "potentially active".

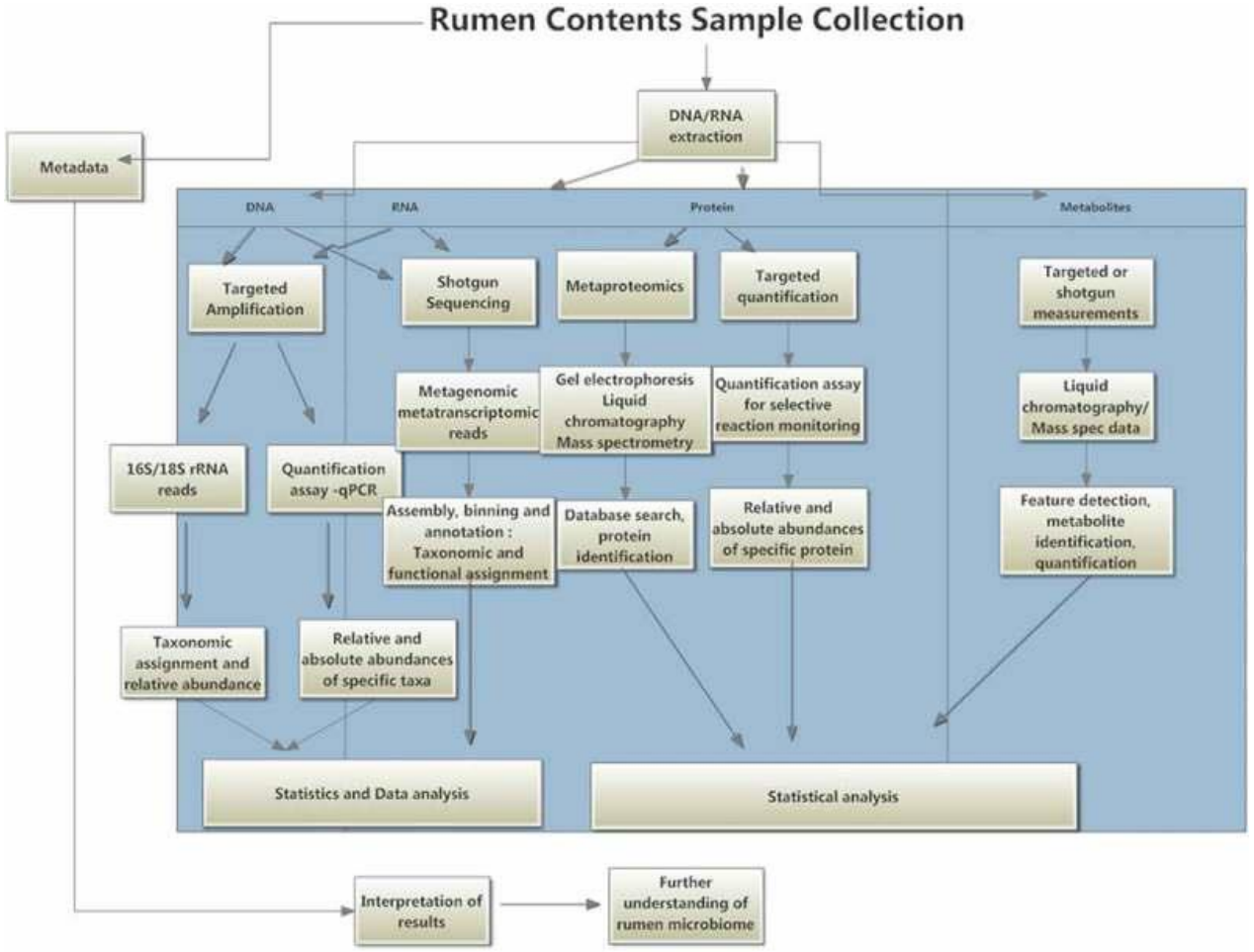


Figure 5.1: Flow chart of potential methodologies and techniques used in rumen microbiota studies adapted from Matthews, et al. (2019).

Nutrition plays a large role in dictating the structure of the microbiome affecting species abundance, function and metabolism (Matthews *et al.*, 2019), thereby effecting the degradation and fermentation of substrate, influencing feed efficiency, animal performance and productivity. The nutritional composition of forage and availability of nutrients within forage has been demonstrated to have an effect on microbial communities. Belanche *et al.* (2016) observed the feeding of conserved Timothy grass hay to result in a decrease in the bacterial and an increase in protozoal concentrations in comparison with fresh grass. Huws *et al.* (2014) demonstrated differences in the bacterial communities colonising the stem and leaf fractions of fresh Perennial ryegrass, with the leaf fraction being degraded faster in comparison with the stem fraction of the plant. Belanche *et al.* (2012) demonstrated cattle fed a high fibrous diet in comparison with a high starch diet to have a greater diversity of bacteria and anaerobic fungi and a greater relative abundance of protozoa, anaerobic fungi and methanogens, however no effect was observed on organic matter digestibility. Likewise alterations to community structure were observed when the protein content of the diet was limiting, where a reduction in the diversity of bacteria and anaerobic fungi were observed along with a reduction in the relative abundances of bacteria, protozoa, anaerobic fungi and methanogens with detriment to organic matter digestibility. Therefore great interest resides in understanding how the microbiome interacts with the diet and the effect of different feeds on microbial community structure, activity and metabolism and how nutritional intervention can manipulate the microbiome for increased feed efficiency, improvements in animal performance, product quality and to reduce environmental emissions.

It has previously been demonstrated in cattle that the degradation and fermentation of dietary substrate occurs as a result of a series of colonisation events involving different communities of microbiota (Cheng *et al.*, 1980, McAllister *et al.*, 1994, Edwards *et al.*, 2007, Edwards *et al.*, 2008, Huws *et al.*, 2013, Huws *et al.*, 2014, Piao *et al.*, 2014, Huws *et al.*, 2016, Liu *et al.*, 2016, Mayorga *et al.*, 2016, Belanche *et al.*, 2017, Cheng *et al.*, 2017, Elliott *et al.*, 2018), therefore great interest resides in understanding these patterns. Such studies are often based on samples extracted from the rumen at a given time point giving an insight into the microbial community structure and diversity but not necessarily dynamics. However, from the use of culture independent techniques such as molecular biology and bioinformatic analysis it is possible to identify microbiota that are present in which community, the likely time frame over which they colonise in and insights into their potential function and role in fermentation.

Low moisture feed licks (LMFL) are a commercial feed supplement for extensively grazing ruminants. Research into the effect of the LMFL on rumen function and the microbiome of sheep is limiting. Findings from Chapter 4 suggest that the LMFL potentially increases the degradation of substrate and

has an enhancing effect on rumen fermentation. However, the LMFL demonstrated no effect on the microbial biomass of the total or potential metabolically active solid associated population (SAP), suggesting supplementation to stimulate microbial metabolism but not population size. The studies in Chapter 4 did not investigate the effect of LMFL supplementation on the structure and diversity of microbiota. Therefore the effects on rumen fermentation may be realised by alterations in the composition of the microbial population which may in consequence promote particular metabolic activities such as those associated with fibre breakdown.

5.2 Chapter Aims

The overall aim of this chapter was to investigate the effect of pre-conditioning the rumen to LMFL supplementation, investigating the effect of the supplement on rumen function and the solid associated bacterial population (SABP). Pre-conditioning of the rumen will be conducted by allowing sheep to consume the LMFL supplement to appetite over an adaptation period to prime the rumen.

This chapter consists of two experiments; Experiment 1 an *in-vitro* study and Experiment 2 an *in-sacco* study. Using the *in-vitro* gas production technique Experiment 1 focusses on the effect of pre-conditioning the rumen to the LMFL investigating rumen function and the SABP. Rumen function will be investigated by measuring the degradation of forage and fermentation within the rumen. The community structure of the SABP will be investigated following the co-extraction of DNA and RNA from residual solid samples and sequencing of the bacterial 16s rRNA gene via NGS to gain an insight into the total and potential metabolically active communities. Using the *in-sacco* technique Experiment 2 aims to measure the effect of LMFL supplementation on the rate and extent of the degradation of forage within the rumen.

Experiment 1: The Effect of Pre-conditioning the Rumen to Low Moisture Feed Lick Supplementation on Rumen Function and the Solid Associated Bacterial Population *in-vitro*

Experiment 2: The Effect of Low Moisture Feed Lick Supplementation on the Degradation of Forage *in-sacco*

5.2.1 Hypothesis

The hypotheses of this chapter are detailed below:

H_1: Pre-conditioning the rumen to the LMFL will increase the degradation and fermentation potential of forage as a result of increased microbial activity

H_2: Pre-conditioning the rumen to the LMFL will affect the structure, diversity and potential metabolic activity of the SABP

H_3: Supplementation of the *in-vitro* bottle diet with the LMFL will increase the degradation and fermentation potential of forage as a result of increased microbial activity

H_4: Supplementation of the *in-vitro* bottle diet with the LMFL will have an effect on the structure, diversity and potential metabolic activity of the SABP

H_5: Differences will reside in the structure, diversity and potential metabolic activity of the SABP at 24 and 48 hours

H_6: Differences will reside between the structure and diversity of the total (DNA) and potential metabolically active (RNA) solid associated bacterial population

5.3 Experimental Design

All experiments had ethical approval as described in Chapter 2 section 2.1. Experimental design (Figure 5.2) consisted of a simple cross over design with two periods, with each animal receiving each treatment once. Periods were 42 days in length and consisted of a 37 day adaptation period followed by a 5 day sampling period in which an *in-vitro* (Experiment 1) and *in-sacco* (Experiment 2) experiment took place. Periods were longer than is traditional in animal studies as a result of University closure days over public holidays. The *in-vitro* study started on day 1 of the sampling period and the *in-sacco* study started on day 3 of the sampling period and lasted for a further 2 days.

Figure 5.2: Experimental design for the pre-conditioning of the rumen to the low moisture feed lick

		Period	
		1	**2**
Group	**A**	+LMFL	–LMFL
	B	–LMFL	+LMFL

Six Aberdale x Texel sheep (average live weight 85.1 kg ± 7.9 kg, 2 wethers and 4 ewes) fitted with rumen cannulas (Chapter 2 section 2.1) were split into two groups (A and B) of 3 balanced for live weight and gender. Groups were housed in separate pens bedded down on straw with free access to clean fresh water and a salt lick (Baby Pure, Rockies, Winsford, UK) to account for no sodium in the LMFL. Groups were fed a forage-based diet of Ryegrass hay *ad libitum.* The chemical analysis of the hay was conducted as described in Chapter 2 section 2.6 to determine the dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), crude protein (CP) and ash content (Table 5.1). Experimental treatments consisted of 1) Control, no supplement (-LMFL) and 2) Treatment, LMFL supplement (+LMFL). The LMFL supplement provided was Crystalyx® Extra High Energy (Caltech-Crystalyx UK, Siloth, UK) as described in Chapter 2 section 2.2.1, in which a 22.5 kg tub was placed in the treatment groups pen for communal access on an *ad-libitum* basis. All sheep were observed to have consumed the LMFL on a daily basis based on the presence of molasses around their mouths.

Table 5.1: Chemical analysis of Ryegrass hay

Dry matter (g/ kg)	899
Organic matter (g/ kg DM)	910
Neutral detergent fibre (g/ kg DM)	773
Acid detergent fibre (g/ kg DM)	482
Crude protein (g/ kg DM)	80.3
Ash (g/ kg DM)	84.0

5.3.1 Supplement Consumption and Live weight

Sheep within groups were housed in a communal pen therefore it was not possible to accurately measure individual daily forage intakes. It was also not possible to measure forage intake on a group basis due to forage getting caught in the straw and spread around the pen which would have led to an underrepresentation of the total daily forage intake. Consumption of the LMFL was measured daily on the last 7 days before the sampling period to estimate the average daily intake of the supplement. The last 7 days of the period were selected so that sheep had acclimatised to having the LMFL as part of their diets. Moreover, it was expected that sheep during the first week of being introduced to the LMFL would indulge and over-consume it as a result of its high palatability. Individual sheep live weights were recorded at the start and end of each period to determine if the LMFL had any effect on

live weight. This was conducted using a portable weigh crate connected to an electronic weigh head as described in Chapter 3 section 3.3.5.

5.3.2 Experiment 1: *In-vitro* Study

Experiment 1 consisted of an *in-vitro* gas production experiment with batch culture design, similar to that conducted in Chapter 4 and as described in Chapter 2 section 2.2. The gas production experiment took place on days 1-2 of the sampling period. Experimental design (Figure 5.3) consisted of a 2 x 2 x 2 factorial design. Factor 1 consisted of the collection of rumen fluid (RF) from sheep supplemented without (RF-) and with (RF+) the LMFL as described in section 5.3 to see if pre-conditioning the rumen to the LMFL had an effect on rumen function and/ or the SABP. Factor 2 consisted of an *in-vitro* bottle diet (BD) of Ryegrass hay supplemented without (BD-) and with (BD+) the LMFL to investigate the effect of a flat rate dose of the LMFL on rumen function and the SABP. Factor 3 consisted of two time points 24 and 48 hours for the sacrificial harvest of bottles. Bottles containing no substrate were included in experimental design to correct for the fermentation of endogenous substrate present within rumen fluid as recommended by Carro *et al.* (2005). Three technical replicates of each treatment were included in the experimental design resulting in 9 bottles per sheep, totalling 54 bottles for the experiment.

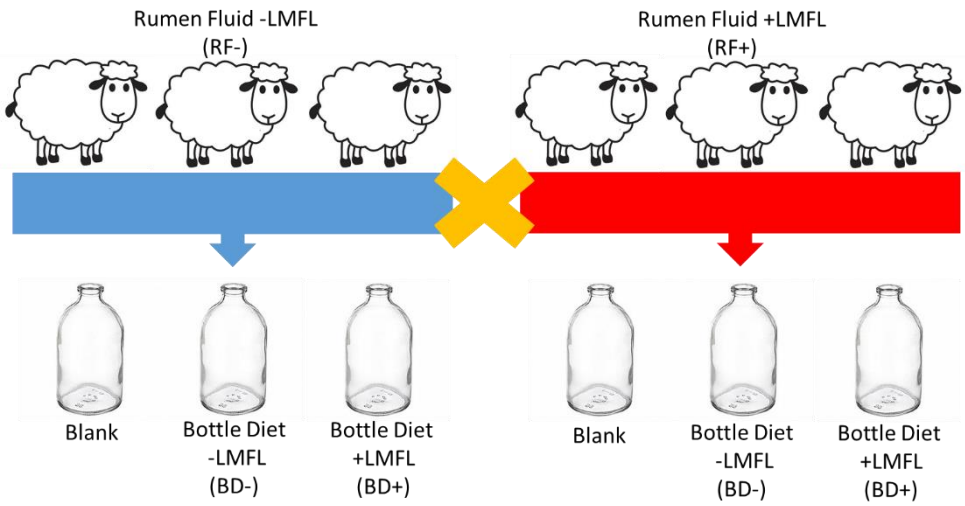


Figure 5.3: Experimental design highlighting experimental factors, rumen fluid (RF) collected from sheep whose diets were not supplemented (RF-) or were supplemented (RF+) the low moisture feed lick (LMFL) and the *in-vitro* bottle diet (BD) of Ryegrass hay in the absence (BD-) and presence (BD+) of the LMFL supplement. Blank bottles are an experimental control of no substrate and no supplement.

In-vitro experimental set up was conducted as described in Chapter 4 and in Chapter 2 section 2.2.4. Fresh Ryegrass hay (Table 5.1) was chopped to 1-2 cm and approximately 0.72 g (0.80 g DM), weighed into 120 ml Wheaton bottles along with 0.14 g of the LMFL supplement where appropriate. The quantity of the LMFL was formulated into the diet based on the estimated average daily consumption of forage (DM) by the sheep (based on 2% of the groups average live weight) along with the average recorded daily consumptions of the LMFL by each group. The ratio of forage to was then scaled down to fit with bottle capacity as conducted by Mayorga *et al.*, 2016.

On the morning of the experiment rumen fluid was collected from the rumen of 6 sheep and as described in Chapter 2 section 2.2.2. Rumen fluid was combined with a phosphate bicarbonate buffer to produce a 1:9 v/v inoculum as described in Chapter 2 section 2.2.3. Bottles were inoculated with 72 ml of the inoculum and stored in an upright standing incubator set at 39^{o}C for the duration of the experiment.

5.3.3 Experiment 1: *In-vitro* Study – Rumen Function

Rumen function was investigated by measuring the products of fermentation; the volume of gas produced and the molar concentrations of the volatile fatty acids (VFAs) and ammonia. Gas pressure was measured from the headspace of bottles at time intervals 1, 2, 4, 6, 8, 10, 12, 18, 24, 30, 36 and 48 hours post inoculation and the cumulative volume of gas calculated as described in Chapter 2 section 2.2.5. At 24 hours one repetition of each treatment for each sheep was subjected to sacrificial harvest and the remaining two bottles harvested at 48 hours as described in Chapter 2 section 2.2.7. During harvest a sub-sample of the liquid fraction was recovered and the appropriate amount of inoculum prepared to determine the molar concentration of the VFAs and ammonia as described in Chapter 2 sections 2.5.2 and 2.5.3. The solid fraction was immediately placed onto pre-weighed labelled foil, wrapped and flash frozen in liquid-N. Samples were placed immediately onto a freeze drier and the percentage of forage dry matter that had degraded calculated as described in Chapter 2 section 2.6.2.

5.3.4 Experiment 1: *In-vitro* Study – 16s rRNA Gene Sequencing

Bacteria from the SABP were identified via next generation sequencing (NGS) using the Ion Torrent Personal Genome Machine (Life Technologies, Thermo Fisher, New Hampshire, USA) and their relative abundances in samples quantified as described in Chapter 2 section 2.10. DNA and RNA were co-extracted from freeze dried residual solid samples for the identification of the total bacterial community (DNA) and the potential metabolically active bacterial community (RNA) as described in Chapter 2 section 2.10.1. To ensure that DNA and RNA had both been successfully extracted and in

sufficient concentrations and quality for amplification, nucleotides within samples were quantified via fluorescence using a Qubit fluorimeter (Invitrogen, ThermoFisher Scientific, New Hampshire, USA) with specific probes for DNA and RNA as described in Chapter 2 section 2.10.2. The V1-V2 hypervariable region of the bacterial 16S rRNA gene was targeted and amplified via the Polymerase Chain Reaction (PCR) as described in Chapter 2 section 2.10.3 for DNA and in Chapter 2 section 2.10.4 for RNA. PCR products were identified via gel electrophoresis as described in Chapter 2 section 2.10.5, purified using Agencourt AMPure XP solid-phase reversible immobilization paramagnetic bead technology (Beckman Coulter, USA) as described in Chapter 2 section 2.10.6 and the concentration of nucleic acids in samples quantified via spectrophotometry using an Epoc Micro-Volume Spectrophotometer (BioTek, Winooski, USA) as described in Chapter 2 section 2.9.3.

Two separate libraries were prepared, one for DNA and the other for RNA as described in Chapter 2 section 2.10.7. Briefly, samples were pooled based on the concentration of nucleic acids within samples to achieve an equi-molar concentration of nucleic acids in each library. Once pooled, libraries underwent a clean up to remove base pairs of < 250 bp and > 700 bp using the double sized selection technique using Agencourt AMPure XP solid-phase reversible immobilization paramagnetic bead technology (Ampure XP, Beckman Coulter, California, USA). To check the success of the clean-up and to determine the molar concentration of nucleic acids in each library for chip loading, libraries were analysed via chip based capillary electrophoresis using a Bioanlayser 2100 (Aligent Technologies, Santa Clara, USA). Libraries were diluted to achieve a molar concentration of 50 pM before undergoing an emulsion PCR, an enrichment procedure and for loading onto a 316v2 (100 Mbp) microchip (Life Technologies, Thermo Fisher, New Hampshire, USA) by an IonChef (Life Technologies, Thermo Fisher, New Hampshire, USA). Chips were then loaded into an Ion Torrent PGM (Life Technologies, Thermo Fisher, New Hampshire, USA) for sequencing of nucleic acids as described in Chapter 2 section 2.10.8.

Analysis of sequencing data was conducted using a bioinformatics pipeline described in Chapter 2 section 2.10.9. The potential function of bacteria associated with carbohydrate and protein metabolism was predicted using CowPI (Wilkinson *et al.*, 2018) as described in Chapter 2 section 2.11.

5.3.5 Experiment 2: *In-sacco* Study

The *in-sacco* study was conducted on days 3-5 of the sampling period. As mentioned in section 5.3 sheep were fed a diet of Ryegrass hay *ad-libitum*. Experimental design consisted of two diets being fed; 1) Ryegrass hay *ad libitum* plus no supplement (-LMFL) and 2) Ryegrass hay *ad libitum* plus the LMFL supplement (+LMFL), which was Crystalyx® Extra High Energy as described in section 5.3.

Six nylon bags filled with fresh Ryegrass hay (Table 5.1) chopped to 1-2 cm were prepared and incubated in the rumens of sheep for 2, 4, 6, 8, 12 and 24 hours using the dual exchange technique (Figure 5.4) as described in Chapter 2 section 2.3.1. Approximately 8.9 g (8 g DM) of forage was weighed into nylon bags and incubated in the rumen for 24 and 48 hours on days 1-2 of the *in-sacco* study, followed by 4.4 g (4g DM) of forage for 2, 4, 8 and 12 hours on day 3 of the experiment. Due to the size of the cannula lumen and limitations in rumen capacity no replicates were made aside from at 2 and 24 hours, which were repeated using the dual exchange system (Figure 5.4) as described in Chapter 2 section 2.3.1.

On removal from the rumen, bags were washed and processed for freeze drying as described in Chapter 2 section 2.3.2. The dry matter degradation of forage within the bags was calculated as described in Chapter 2 section 2.6.2. Data for the percentage of forage that had degraded over time was modelled using the exponential equation $Y = a+b\ (1-e^{(-ct)})$ by Ørskov & McDonald (1979) to determine the kinetics of degradation as described in Chapter 2 section 2.2.5.

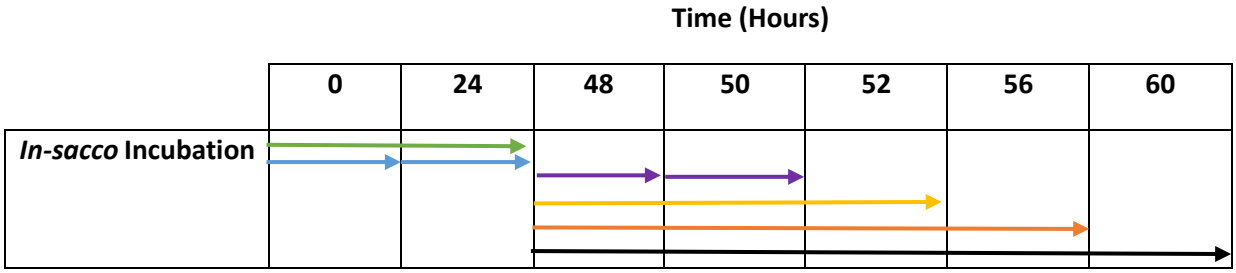


Figure 5.4: Dual exchange system for *in-sacco* incubations. Coloured arrows represent the duration of bags in the rumen, green = 48, blue= 24, purple = 2, yellow = 4, orange =8 and black = 12 hours.

5.4 Statistics

5.4.1 Experiment 1: *In-vitro* Study

The effect of LMFL supplementation on the temporal cumulative volume of gas was analysed via repeated measures analysis of variance (ANOVA) in Genstat (19th edition), with rumen fluid and substrate as treatment factors, time as the repeated measure and blocking for sheep and period. The effect of LMFL supplementation on fermentation products VFAs and ammonia was measured using multiple analysis of variance (MANOVA) with rumen fluid, bottle diet and time as factors and blocking for sheep and period. Significance was regarded at $p < 0.05$.

Normalised relative sequence abundance matrices of OTUs from the total bacterial community (referred to hereafter as DNA) and the potential metabolically active bacterial community (referred to hereafter as RNA), were subjected to a square root transformation to reduce the effect of dominant taxa in statistical tests. These transformed matrices were used in the following analyses.

Principal component ordination analysis (PCoA) with 95% ellipses and non-parametric Permutational analysis of variance (PerMANOVA) was carried out using the Bray-Curtis distance matrix with 999 random permutations for DNA and RNA in Palentological Statistics Software Package for Education and Data Analysis 325 (PAST) (Hammer *et al.*, 2001). Significance was regarded at $p < 0.05$.

Alpha diversity of DNA and RNA was using Inverse Simpson and Shannon diversity indices. Data were analysed via MANOVA with rumen fluid and substrate as treatment factors, time as the repeated measure and blocking for sheep and period as described above. Significance was regarded at $p < 0.05$.

Differences in the relative abundance of OTUs identified to phyla and genera level within phyla were analysed via MANOVA for DNA and RNA. Significance was regarded at $p < 0.05$.

The potential function of bacteria as predicted using CowPI (Wilkinson *et al.*, 2018) was measured via principal component analysis (PCA) using the Euclidean distance matrix with 999 random permutations with 95% ellipses. PerMANOVA was carried out as described above. Data was transformed via log(2) transformation and the fold change of potential gene expression calculated and results presented as a heat map with statistical analysis conducted via MANOVA.

5.4.2 Experiment 2: *In-sacco* Study

The effect of LMFL supplementation on the temporal dry matter degradation of forage was analysed via repeated measures ANOVA in Genstat (19th edition), with rumen fluid and substrate as treatment factors, time as the repeated measure and blocking for sheep and period. Significance was regarded at $p < 0.05$.

5.5 Results

5.5.1 Supplement Consumption and Live weight

The estimated daily consumption of the LMFL and live weight were measured. Although groups were balanced for live weight and gender group B (320g ± 36.76g) consumed more of the LMFL in comparison with group A (373g ± 54.68g), however there was no significant difference between the consumption of the two groups. Live weight was measured at the start and end of each period. There was no difference between the live weights of each group at the start and end of each period (Table

5.2). Both groups gained weight between the start and end of periods suggesting positive energy balance, however there was no difference between the live weight gained by each group.

Table 5.2: Effect of low moisture feed lick supplementation on the live weight of sheep

Live Weight (kg)	-LMFL	+LMFL	sed	p-Value
Start	86.4	85.6	2.50	0.764
End	87.5	88.2	1.75	0.709
Gain/ Loss	1.10	2.60	3.26	0.668

- Absence of the LMFL
+ Presence of the LMFL

5.5.2 Experiment 1: *In-vitro* Study - Rumen Function

The effect of LMFL supplementation on rumen function was investigated by measuring the degradation of forage and fermentation products generated from *in-vitro* cultures. Pre-conditioning the rumen to the LMFL had no effect on the percentage of forage that had degraded at 24 or 48 hours (Table 5.3). Likewise, there was no difference in the percentage of substrate dry matter that had degraded when the *in-vitro* bottle diet was supplemented with the LMFL. Time had an increasing effect on the dry matter degradation of forage ($p<0.001$) where a greater proportion of the forage had been degraded at 48 hours in comparison with 24 hours. There were no significant interactions between factors aside from pre-adaptation of rumen and time ($p=0.028$).

The volume of gas produced over 48 hours was measured and revealed pre-adaptation of the rumen to the LMFL to have no effect on the volume of gas produced (Figure 5.5). Likewise, pre-adaptation of the rumen to the LMFL had no effect on the cumulative volume of gas produced per gram of substrate dry matter (Figure 5.6). Supplementation of the bottle diet (Ryegrass hay) with the LMFL increased the cumulative volume of gas produced ($p<0.001$). Likewise, LMFL supplementation had an increasing effect on the cumulative volume of gas produced per gram of substrate dry matter ($p<0.001$). There were no significant interactions between factors aside for the bottle diet and time ($p<0.001$).

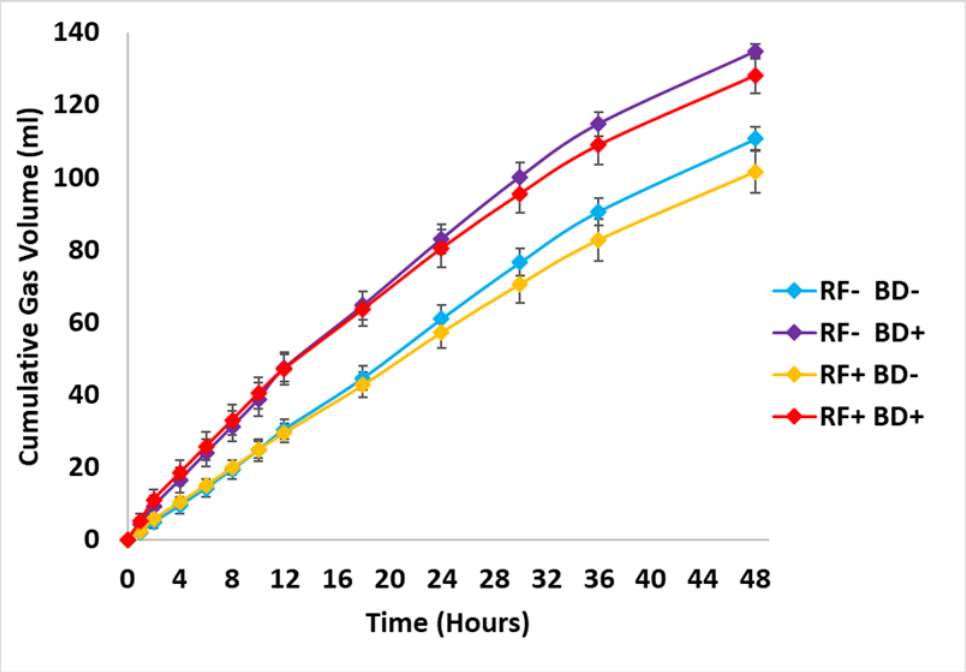


Figure 5.5: Effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with the LMFL on the cumulative volume of gas (ml) produced over 48 hours (T). Where, - represents the absence of the LMFL and + represents the presence of the LMFL. Data points are means from all sheep (n=6) and error bars are the standard error of the mean. RF ($p=0.740$), BD ($p<0.001$), T ($p<0.001$), RF x BD ($p=0.672$), RF x T ($p=0.082$), BD x T ($p<0.001$), RF x BD x T ($p=0.966$).

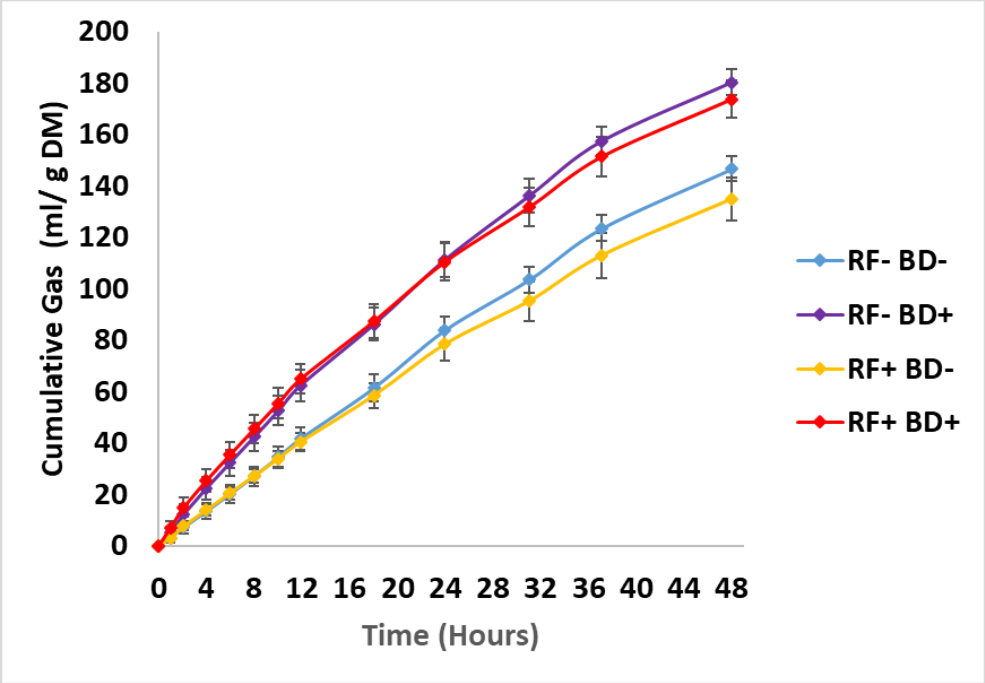


Figure 5.6: Effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with the LMFL on the cumulative volume of gas (ml) produced per gram of dietary dry matter over 48 hours (T). Where, – represents the absence of the LMFL and + represents the presence of the LMFL. Data points are means from all sheep (n=6) and error bars are the standard error of the mean. RF ($p=0.510$), BD ($p<0.001$), T ($p<0.001$), RF x BD ($p=0.398$), RF x T ($p=0.200$), BD x T ($p<0.001$), RF x BD x T ($p=0.966$).

The molar concentration of the fermentation products; VFAs and ammonia were measured (Table 5.3). Pre-adaptation of the rumen to the LMFL had no effect on the molar concentration of total VFAs. However, supplementation of the *in-vitro* bottle diet with the LMFL had the effect of increasing the molar concentration of total VFAs ($p<0.001$). Time had an increasing effect on the total molar concertation of VFAs ($p<0.001$). There were no interactions between factors.

Pre-adaptation of the rumen to the LMFL had no effect on the molar concentrations of; acetate, propionate or the branched chain VFAs (valerate, iso-valerate, iso-butyrate). However, supplementation displayed a tendency to decrease the molar concentration of butyrate ($p=0.0058$). Supplementation of the *in-vitro* bottle diet with the LMFL had the effect of increasing the molar concentrations of; acetate, propionate, butyrate and the branched chain VFAs ($p<0.001$). Time had an increasing effect on the molar concentrations of all individual VFAs ($p<0.001$). There were no significant interactions between factors.

The ratio of acetate to propionate (A:P) was measured as an indication of the potential pathway of fermentation as described in Chapter 2 section 2.5.2 (Table 5.3). Pre-adaptation of the rumen to the LMFL had no effect on A:P. However, supplementation of the *in-vitro* bottle diet with the LMFL had the effect of increasing A:P ($p<0.001$). Time had no effect on A:P. Interactions between factors were observed for pre- conditioning the rumen to the LMFL and time ($p=0.019$).

The molar concentration of ammonia was measured as a potential indicator of protein metabolism within the rumen as described in Chapter 2 section 2.5.3. Pre-adaptation of the rumen to the LMFL and supplementation of the *in-vitro* bottle diet with the LMFL had no effect on the molar concentration of ammonia. Time had an increasing effect on the molar concentration of ammonia ($p<0.001$). There were no significant interactions between factors.

Table 5.3: Effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with he LMFL on the degradation of forage and rumen fermentation products at 24 and 48 hours

	RF-	RF+	sed	BD-	BD+	sed	24	48	sed	RF	BD	T	RFxBD	RFxT	BDxT	RFxBDxT
Dry matter degradation (%)	44.0	42.1	2.027	43.3	42.8	0.507	35.1	51.0	0.527	0.414	0.358	<0.001	0.239	0.028	0.903	0.643
Total VFAs (mM)	30.3	28.7	1.123	24.9	34.0	1.089	23.4	35.5	1.865	0.228	<0.001	<0.001	0.423	0.372	0.929	0.370
Acetate (mM)	17.6	16.5	0.793	14.8	19.3	0.679	13.9	20.2	1.001	0.233	<0.001	<0.001	0.416	0.517	0.911	0.418
Propionate (mM)	8.20	8.09	0.224	6.55	9.76	0.261	6.74	9.57	0.605	0.571	<0.001	<0.001	0.140	0.081	0.851	0.350
Butyrate (mM)	2.90	2.74	0.082	2.35	3.35	0.170	2.16	3.53	0.206	0.058	<0.001	<0.001	0.491	0.319	0.533	0.257
Branched Chain (mM)	0.97	0.91	0.064	0.81	1.07	0.058	0.40	1.47	0.072	0.404	<0.001	<0.001	0.832	0.932	0.125	0.346
A:P	2.15	2.08	0.075	1.98	2.26	0.027	2.10	2.13	0.058	0.390	<0.001	0.345	0.317	0.019	0.076	0.438
Ammonia (mM)	4.75	4.85	0.548	4.58	5.03	0.548	3.03	6.58	0.432	0.856	0.416	<0.001	0.697	0.383	0.638	0.951

T = Time

- Absence of the LMFL

+ Presence of the LMFL

5.5.3 Experiment 1: *In-vitro* study - 16s rRNA Gene Sequencing

Identification of bacteria from the solid associated sub-division of the microbial population was conducted via sequencing (NGS) of the 16S rRNA gene from the co-extraction of DNA and RNA from residual forage samples. Sequencing of the 16S rRNA gene from DNA and RNA extracts generated 993360 and 947002 raw sequences respectively. Sequences for RNA were numerically lower in comparison with DNA. This may be due to RNA being fragile and having a higher rate of degradability in comparison with DNA which is fairly robust. Post quality filtering the average number of sequences per sample for DNA extracts resided at 6888 with a mean base pair length of 444 base pairs. Post quality filtering the average number of sequences per sample for RNA resided at 6672 sequences with a mean base pair length of 436 base pairs. Samples with low sequence counts (<0.05%) were excluded from the dataset. Datasets for RNA and DNA were merged and clustered into 502 different operational taxonomic units (OTUs). OTUs within DNA samples resided at 464 different OTUs and OTUs within RNA resided at 388 different OTUs.

The OTU for Chloroplast/ Cyanobacteria was removed from both DNA and RNA datasets due to being associated with dietary substrate rather than a part of the rumen bacterial community. Reasons why the OTU for Chloroplast/ Cyanobacteria was sequenced may have been a result of primer selection. Edwards *et al.* (2007) highlighted the importance of primer design in rumen colonisation studies using 16s rRNA sequencing technology, suggesting the amplification and sequencing of 16s rRNA chloroplast sequences to dominate and as a result inflict limitations on studies when using fresh grass as a dietary substrate. It was thought due to the substrate being hay little amplification of 16s rRNA Chloroplast/ Cyanobacteria sequences would occur as a result of few Chloroplast/ Cyanobacteria being present in conserved forages such as hay and that the primer for the V1/V2 hypervariable region would suffice. The total number of sequences for Chloroplast/ Cyanobacteria was greater in the DNA data set and accounted for 12908 sequences whereas in the RNA dataset 528 sequences were present. This may be expected as RNA is representative of the potential active rumen microbial population and any residual plant transcriptional activity.

The clustering of sequences into OTUs and identification of OTUs from phyla to genus level was conducted using the ribosomal database project (RDP) as described in Chapter 2 section 2.10.9. Not all OTUs were able to be identified as far down as genus level. Based on classification by the RDP 91% of all OTUs identified were unclassified at genera level, 57% at the family level, 30.7% at the order level, 28.3% at the class level and 5.6% at the phyla level. Any unidentified OTUs at the phyla level were placed into the category “unclassified”. OTUs identified within phyla resided at, 237 OTUs for *Firmicutes;* 196 OTUs for *Bacteroidetes*; 29 OTUs for *Proteobacteria*, 6 OTUs for *Spirochaetes,* 28

unclassified OTUs and 1 OTU for *Firmicutes*, *Tenericutes*, *Synergistetes*, *Candidatus Sacharibacteria*, *Lentisphaerae*. *Spirochaetes, Actinobacteria*.

The relative abundance of phyla in the total and potential metabolically active SABP differed significantly for *Bacteroidetes*, *Firmicutes*, *Spirochaetes* and *Tenericutes* ($p<0.05$) (Figure 5.7). The order of the most abundant phyla found in the total SABP was *Fibrobacteres* (44.6 %), *Bacteroidetes* (31.0 %), *Firmicutes* (14.4 %), *Spirochaetes* (6.58 %), *Tenericutes* (1.54 %), other phyla (1.52 %), unclassified phyla (0.39 %) and *Proteobacteria* (0.39 %). The order of the most abundant phyla in the potential metabolically active SABP was Bacteroidetes (52.3 %), *Fibrobacteres* (33.8 %), *Firmicutes* (8.72 %), *Spirochaetes* (3.19 %), Tenericutes (0.*94* %), other phyla (0.94%), unclassified phyla (0.76 %) and *Proteobacteria* (0.21 %). Other phyla consisted of *Candidatus* Saccharibacteria, *Synergistetes* and *Lentisphaerae*.

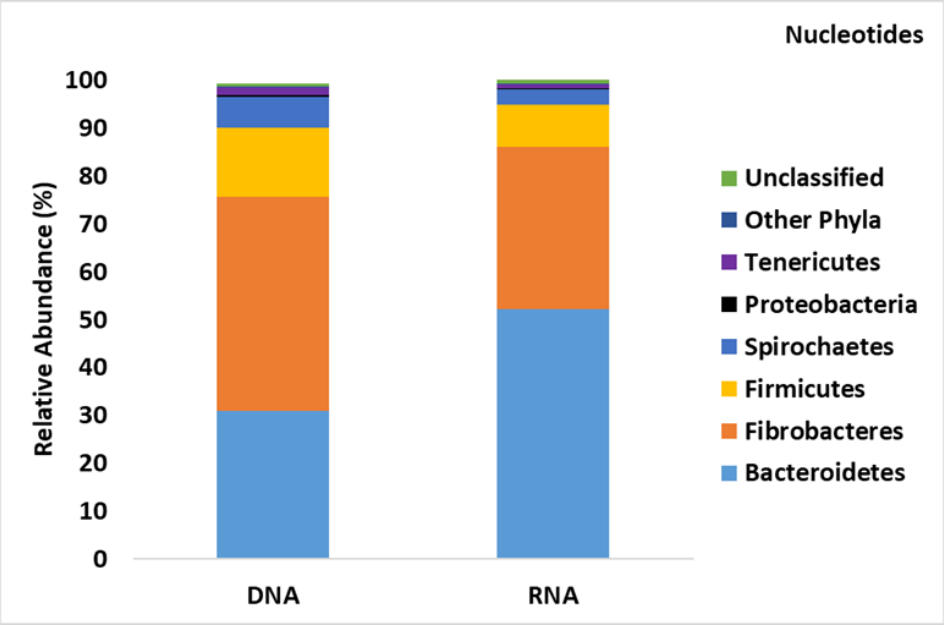


Figure 5.7: Relative abundance of phyla from the total (DNA) and potential metabolically active (RNA) solid associated bacterial population. Other phyla are *Candidatus Saccharibacteria*, *Synergistetes* and *Lentisphaerae*. Bacteroidetes ($p=0.002$), *Fibrobacteres* ($p=0.002$), *Firmicutes* ($p=0.002$), *Spirochaetes* ($p=0.002$), *Proteobacteria* ($p=0.037$), *Tenericutes* ($p=0.008$), Other Phyla ($p=0.660$), Unclassified Phyla ($p=0.177$).

The effect of experimental factors on the relative abundance of bacterial phyla was investigated in the total (Figure 5.8) and potential metabolically active SABP (Figure 5.9). Pre-conditioning the rumen to the LMFL and supplementation of the *in-vitro* bottle diet with the LMFL had no effect on the relative abundance of phyla within the total or potential metabolically active SABP. Time had a significant effect on the relative abundance of certain phyla in both the total and potential metabolically active SABP. In the total SABP time had the effect of increasing the relative abundance of *Firmicutes* (p=0.04), other phyla (p=0.002) and unclassified phyla (p=0.002) decreasing the abundance of *Tenericutes* (p=0.002) and *Spirochaetes* (p=0.002). In the potential metabolically active SABP time had the effect of increasing the relative abundance of *Firmicutes* (p=0.044) and *Protobacteria* (p=0.008) and decreasing the relative abundance of *Spirochaetes* (p=0.016). There were no significant interactions between factors for the total or potential metabolically SABP.

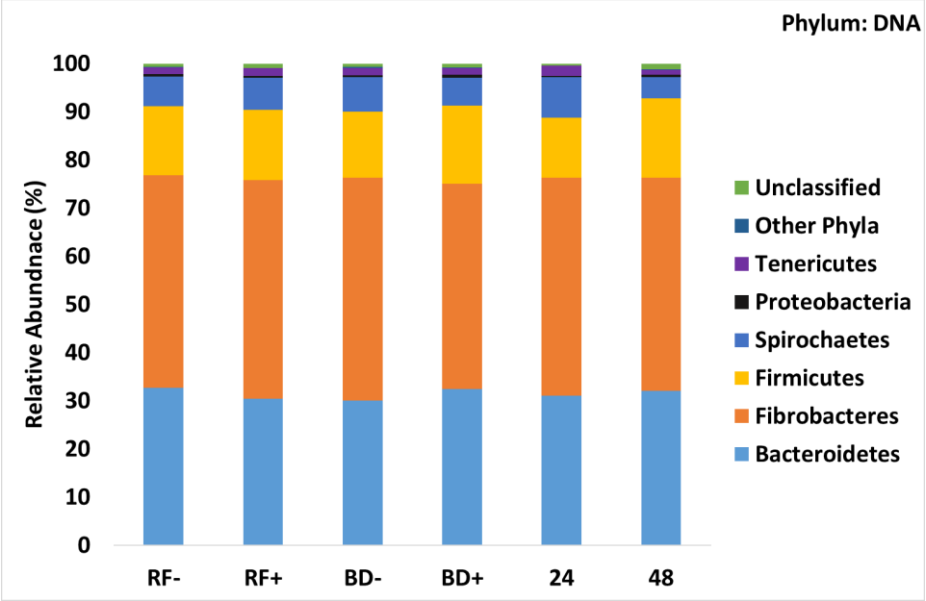


Figure 5.8: Effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL), supplementation of the *in-vitro* bottle diet (BD) with the LMFL and time points 24 and 48 hours on the relative abundance of phyla of the total (DNA) solid associated bacterial population. Where, – represents the absence of the LMFL and + represents the presence of the LMFL.

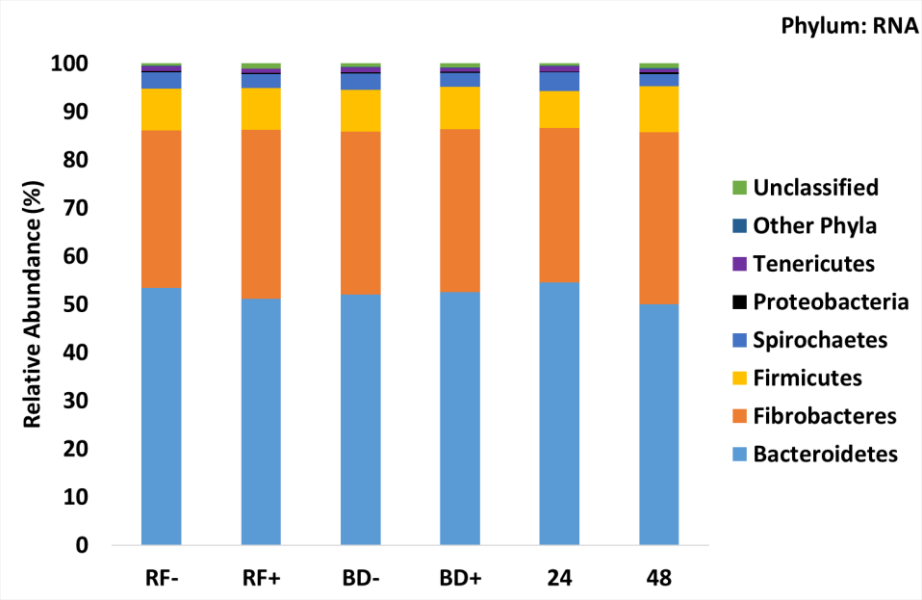


Figure 5.9: Effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL), supplementation of the *in-vitro* bottle diet (BD) with the LMFL and time points 24 and 48 hours on the relative abundance of phyla of the potential metabolically active (RNA) solid associated bacterial population. Where, – represents the absence of the LMFL and + represents the presence of the LMFL.

Principal component ordination analysis (PCoA) was conducted on the OTU table at the genera level to visualise if any differences in the structure of the total (DNA) and potential metabolically active (RNA) SABP resided. Likewise, visualisations were also made for the pre-conditioning of the rumen to the LMFL, supplementation of the *in-vitro* bottle diet with the LMFL and for time points 24 and 48 hours. The PCoA plot generated (Figure 5.10) displayed differential clustering of the total and potential metabolically active SABP and at time points 24 and 48 hours suggesting differences in the community structure to reside. No clear differences were detected within treatments; pre-conditioning of the rumen to the LMFL and the supplementation of the *in-vitro* bottle diet with the LMFL. For better visualisation PCoA plots were generated for the total and potential metabolically active SABP at each time point (Figure 5.11 A-D). However, there were no clear differences within treatments; suggesting the pre-conditioning of the rumen to the LMFL and supplementation of the i*n-vitro* bottle with the LMFL to not have any effect on the structure of the total or potential metabolically active SABP.

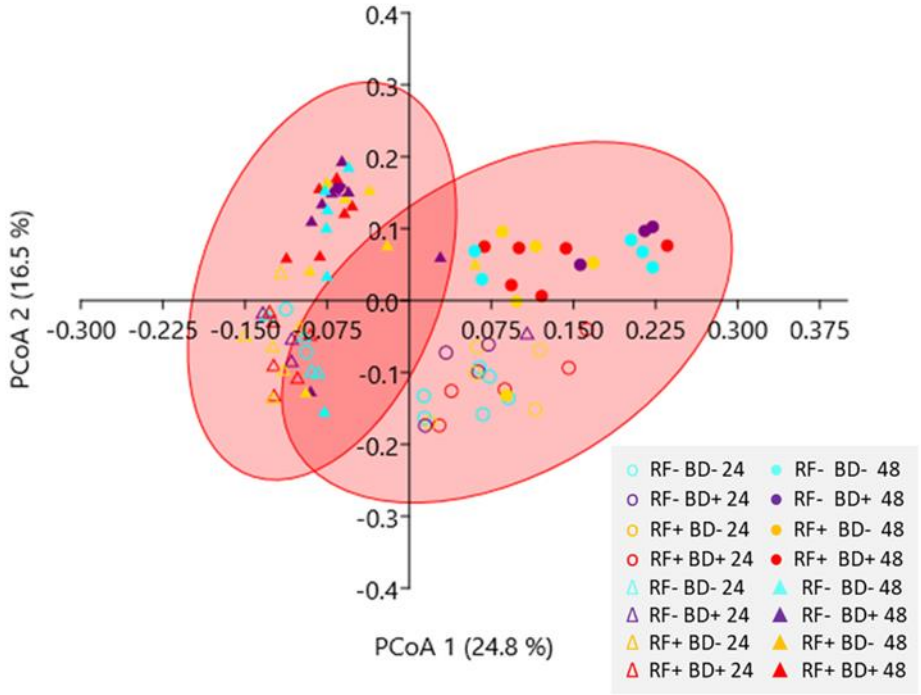


Figure 5.10: Principal component ordination analysis (PCoA) demonstrating the effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with the LMFL at time points 24 (non-filled shapes) and 48 (filled shapes) hours on the structure of the total (DNA;o) and potential metabolically active (RNA;Δ) solid associated bacterial population. Where, – represents the absence of the LMFL and + represents the presence of the LMFL. Red filled ovals represent 95% confidence ellipses for DNA and RNA.

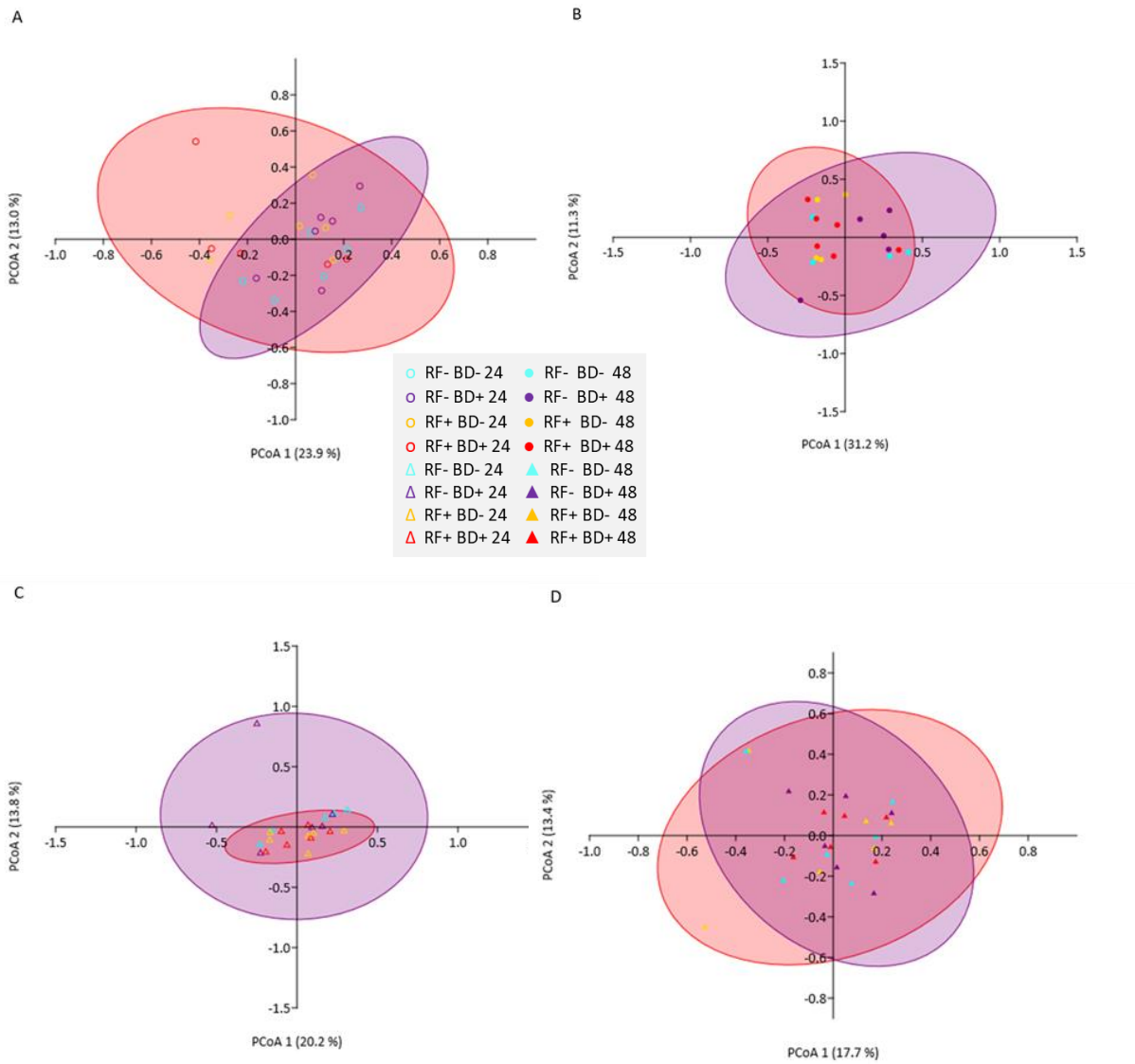


Figure 5.11: Principal component ordination analysis (PCoA) demonstrating the effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with the LMFL at 24 (A,C; non-filled shapes) and 48 (B,D; filled shapes) hours on the structure of the total (A,B; DNA;o) and potential metabolically active (C,D; RNA;Δ) solid associated bacterial population. Where, – represents the absence of the LMFL and + represents the presence of the LMFL. Purple filled ovals represent 95% confidence ellipses for RF- and red filled ovals represent 95% confidence ellipses for RF+.

Permutational analysis of variance (PerMANOVA) was carried out at the genera level and revealed pre-conditioning of the rumen to the LMFL and supplementation of the *in-vitro* bottle diet with the LMFL to have no effect on the structure of the total or potential metabolically active SABP (Table 5.4). Time had a significant effect on the community structure (p=0.0001) for both the total and potential metabolically active SABP. There were no interactions between factors. Differences in community structure were observed between sheep (p<0.001), the total and potential metabolically active SABP (p<0.001) and the two periods (p<0.01).

Table 5.4: Permutational analysis of variance for the effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL), supplementation of the *in-vitro* bottle diet (BD) with the LMFL and time (T) on the structure of the total (DNA) and potential metabolically active (RNA) solid associated bacterial population

	DNA	RNA
RF	0.317	0.111
BD	0.321	0.530
T	0.001	0.001
RF x BD	0.998	0.969
RF x T	0.269	0.348
BD x T	0.781	0.935

Analysis of the data by PerMANOVA as described above did not take into account the effect of experimental factors within each time point. Therefore, additional analysis of the data via PerMANOVA was carried out at each time point (Table 5.5). PerMANOVA revealed at 24 hours pre-conditioning of the rumen to the LMFL had an effect on the structure of the total (p=0.049) and potential metabolically active (p=0.047) SABP. However, supplementation of the *in-vitro* bottle diet with the LMFL had no effect on the structure of the total or metabolically active SABP. There were no interactions between factors. At 48 hours pre-conditioning of the rumen to the LMFL had an effect on the structure of the total (p=0.045) and metabolically active (p=0.015) SABP. Supplementation of the *in-vitro* bottle diet with the LMFL had no effect on the structure of the SABP of the total or potential metabolically active SABP. There were no interactions between factors.

Table 5.5: Permutational analysis of variance demonstrating the effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with the LMFL on the structure of the total (DNA) and potential metabolically active (RNA) solid associated bacterial population at 24 and 48 hours

	DNA	RNA
24 Hours		
RF	0.049	0.047
BD	0.994	0.876
RF x BD	0.738	0.563
48 Hours		
RF	0.045	0.015
BD	0.748	0.855
RF x BD	0.817	0.99

Alpha diversity using the Inverse Simpson and Shannon indices was measured as an indicator of the diversity of the total and potential metabolically active SABP. The values obtained across the study are indicative of the presence of a diverse community to be present across treatments. This is perhaps a result of the dietary substrate being hay. Belanche *et al.* (2016), demonstrated the rumen bacterial community colonising Ryegrass hay to have greater alpha diversity in comparison with fresh forage attributed to the a more diverse bacterial community being required to degrade conserved forage due to a high content of lignocellulolytic material and a low content of rapidly degradable carbohydrates. There was no difference between the alpha diversity in either indices for the total or potential metabolically active SABP. This suggests the bacterial diversity and abundance of the different species to be similar across the populations (Table 5.6).

Table 5.6: The alpha diversity of the total (DNA) and potential metabolically active (RNA) solid associated bacterial population

Alpha diversity index	DNA	RNA	sed	p-Value
Inverse Simpson	13.6	14.1	0.831	0.545
Shannon	3.48	3.41	0.046	0.119

The effect of pre-conditioning of the rumen to the LMFL, supplementation of the *in-vitro* bottle diet with the LMFL and time on the alpha diversity within the total and potential metabolically active SABP

was investigated (Table 5.7). Pre-conditioning the rumen to the LMFL and supplementation of the *in-vitro* bottle diet with the LMFL had no effect on the alpha diversity in either indices in the total or potential metabolically active SABP. Time had a significant effect on the alpha diversity of the SABP in the Shannon index in both the total ($p=0.031$) and potential metabolically active SABP where diversity increased with time. There were no significant interactions between factors aside from the pre-conditioning of the rumen to the LMFL and time ($p=0.046$) in the Inverse Simpson index for the potential metabolically active SABP.

Table 5.7: Effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with LMFL on the alpha diversity of the total (DNA) and potential metabolically active (RNA) solid associated bacterial population at 24 and 48 hours (T)

	RF-	RF+	sed	BD-	BD+	sed	24	48	sed	RF	BD	T	RFxBD	RFxT	BDxT	RFxBDxT
Inverse Simpson																
DNA	13.4	14.1	1.504	13.0	14.6	1.316	12.8	14.7	1.146	0.629	0.280	0.225	0.976	0.156	0.148	0.458
RNA	13.7	14.5	1.119	14.0	14.1	0.745	13.8	14.4	0.745	0.501	0.898	0.417	0.914	0.046	0.542	0.772
Shannon																
DNA	3.47	3.52	0.078	3.44	3.54	0.046	3.40	3.58	0.046	0.344	0.219	0.031	0.583	0.067	0.345	0.163
RNA	3.48	3.38	0.044	3.40	3.42	0.045	3.37	3.45	0.045	0.371	0.673	0.088	0.962	0.363	0.557	0.509

- Absence of the LMFL

+ Presence of the LMFL

OTUs with high relative abundances are most likely to be the dominant bacteria associated with the degradation and fermentation of dietary components in the rumen. Therefore, investigations were made into the top 20 OTUs discovered in the total (Table 5.8) and potential metabolically active (Table 5.9) SABP.

There were similarities in the top 20 OTUs identified in the total and potential metabolically active SABP. Such OTUs included; *Fibrobacter*; OTU 3; *Paraprevotella*; OTU 5; OTU 7; OTU 19; OTU 74; *Treponema*; OTU 4; *Prevotella*; *Butyrivibrio*; *Ruminococcus*; OTU 580; *Anaeroplasma*; OTU 285; OTU 26; OTU 531 and OTU 138. *Fibrobacter* was the most abundant genus in both the total and potential metabolically active SABP. This is perhaps unsurprising regarding *Fibrobacter*s being renowned for having the ability to degrade ligno-celluloltyic material in the herbivore gastrointestinal tract (Ransom-Jones *et al.*, 2012). Likewise, OTU 3 and *Paraprevotella* were in the top 5 most abundant OTUs in both populations. These OTUs belong to the family *Prevotellaceae* which has previously been identified as a major bacterial family within digesta (Belanche *et al.*, 2016, Carreño *et al.*, 2019).

Investigations were made into the effect of pre-conditioning the rumen to the LMFL, supplementation of the *in-vitro* bottle diet with the LMFL and time on the relative abundances of the top 20 OTUs in the total (Table 5.8) and potential metabolically active SABP (Table 5.9). In the total SABP (Table 5.8) pre-conditioning the rumen to the LMFL had no effect on the relative abundance of the top 20 OTUs, however there was a tendency for supplementation to decrease the relative abundance of OTU 22 ($p=0.057$). Supplementation of the *in-vitro* bottle diet with the LMFL had an the effect of increasing the relative abundances of OTU 4 ($p=0.012$) and OTU 531 ($p=0.006$) and a tendency to decrease the relative abundance of *Treponema* ($p=0.064$) and *Paraprevotella* ($p=0.057$). Time had the greatest effect on the relative abundance of OTUs where the relative abundance of OTU 3 ($p<0.001$), OTU 4 ($p<0.001$), *Schwartzia* ($p<0.001$), OTU 16 ($p<0.001$), OTU 138 ($p<0.001$), OTU 531 ($p<0.001$), OTU 19 ($p<0.001$) and OTU 22 ($p=0.002$) increased with time and the relative abundance of *Treponema* ($p<0.001$), *Paraprevotella* ($p<0.001$), *Anaeroplasma* ($p<0.001$), OTU 25 ($p=0.0085$), OTU 74 ($p=0.006$), OTU 26 ($p<0.001$) and *Pseudobutyrivibrio* ($p=0.015$) decreased with time. Interactions between factors were observed for the pre-conditioning of the rumen with the LMFL and time for *Treponema* ($p=0.038$), OTU 9 ($p=0.014$), *Prevotella* (0.035), *Anaeroplasma* ($p=0.004$), OTU 26 ($p=0.016$).

In the potential metabolically active SABP (Table 5.9) pre-conditioning of the rumen with the LMFL had no effect on the relative abundance of OTUs, aside from OTU 19 ($p=0.052$) in which there was a tendency for the relative abundance to decrease with supplementation. LMFL supplementation of the *in-vitro* bottle diet had the effect of increasing the relative abundance of OTU 7 ($p=0.036$) and a decreasing effect on the relative abundance of *Paraprevotella* ($p=0.034$) and OTU 531 ($p=0.053$). Time

had the greatest effect on the relative abundance of OTUs where the relative abundance of OTU 19 ($p<0.001$), *Treponema* ($p=0.004$), *Prevotella*, ($p=0.005$), *Butyrivibrio* ($p<0.001$), *Rumionococcus* ($p=0.025$), OTU 285 ($p<0.001$) and OTU 138 ($p<0.001$) increased with time and OTU 3 ($p<0.001$), *Paraprevotella* ($p<0.001$), OTU 74 ($p<0.001$), OTU 580 ($p<0.001$), *Anaeroplasma* ($p<0.001$), OTU 26 ($p<0.001$), OTU 87 ($p<0.001$), OTU 452 ($p<0.001$) decreased with time. Interactions between factors were observed for the pre-conditioning of the rumen to the LMFL for *Provetella* ($p=0.018$), *Ruminococcus* ($p<0.001$) and for the *in-vitro* bottle diet and time for OTU 285 ($p=0.040$).

Table 5.8: The effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with the LMFL on the top 20 operational taxonomic units (OTUs) for the total (DNA) solid associated bacterial population at 24 and 48 hours

Genus	RF-	RF+	sed	BD-	BD+	sed	24	48	sed	RF	BD	T	RF x BD	RF x T	BD x T	RF x BD x T
Fibrobacter	43.9	45.3	2.484	46.2	42.9	2.852	45	44.1	2.770	0.599	0.259	0.746	0.626	0.309	0.178	0.689
Treponema	6.07	6.35	0.743	6.99	5.42	0.807	8.31	4.11	0.842	0.720	0.064	<0.001	0.595	0.038	0.400	0.811
Paraprevotella	4.47	4.18	1.028	4.72	3.93	0.396	6.06	2.60	0.398	0.792	0.057	<0.001	0.977	0.139	0.718	0.690
OTU 3	4.56	3.6	0.540	4.29	3.87	0.471	5.69	2.47	0.461	0.150	0.381	<0.001	0.357	0.459	0.716	0.216
OTU 4	3.11	3.99	0.988	3.16	3.94	0.285	2.99	4.11	0.303	0.420	0.012	<0.001	0.692	0.744	0.878	0.109
OTU 9	3.22	2.38	1.146	2.34	3.26	0.699	2.54	3.06	0.740	0.508	0.202	0.464	0.797	0.014	0.145	0.670
Prevotella	1.60	2.67	0.649	2.14	2.13	0.390	2.45	1.81	0.368	0.175	0.979	0.114	0.866	0.352	0.487	0.411
Anaeroplasma	1.32	1.66	0.568	1.62	1.35	0.227	2.10	0.88	0.229	0.579	0.245	<0.001	0.452	0.035	0.356	0.744
Schwartzia	1.79	1.21	0.685	1.18	1.82	0.390	0.61	2.38	0.426	0.441	0.111	<0.001	0.409	0.004	0.388	0.740
OTU 7	1.53	0.67	0.749	0.71	1.49	0.481	1.14	1.06	0.469	0.316	0.114	0.882	0.204	0.824	0.801	0.892
OTU 25	1.00	1.22	0.224	1.15	1.07	0.250	1.34	0.89	0.232	0.385	0.746	0.085	0.695	0.380	0.761	0.818
OTU 16	1.13	1.52	0.522	1.40	1.25	0.419	0.39	2.25	0.395	0.499	0.733	<0.001	0.388	0.958	0.574	0.299
OTU 5	1.39	0.67	0.797	0.60	1.45	0.483	1.06	0.99	0.482	0.416	0.089	0.885	0.103	0.576	0.638	0.875
OTU 74	1.08	0.85	0.396	1.06	0.87	0.234	1.32	0.61	0.228	0.603	0.420	0.006	0.780	0.072	0.835	0.869

OTU 138	1.04	0.81	0.321	0.84	1.01	0.210	0.22	1.63	0.200	0.324	0.401	<0.001	0.511	0.354	0.325	0.634
OTU 531	0.76	0.86	0.245	0.70	0.917	0.073	0.66	0.95	0.082	0.697	0.006	<0.001	0.542	0.123	0.559	0.080
OTU 26	0.78	0.70	0.399	0.65	0.83	0.127	1.04	0.44	0.140	0.848	0.183	<0.001	0.764	0.016	0.129	0.358
OTU 19	1.06	0.40	0.412	0.69	0.77	0.291	0.37	1.09	0.283	0.182	0.784	0.021	0.342	0.133	0.592	0.870
Pseudobutyrivibrio	0.51	0.88	0.194	0.71	0.684	0.164	0.91	0.48	0.151	0.130	0.869	0.015	0.779	0.909	0.528	0.757
OTU 22	0.87	0.63	0.090	0.67	0.836	0.149	0.49	1.02	0.164	0.057	0.276	0.002	0.923	0.002	0.883	0.999

- Absence of the LMFL
+ Presence of the LMFL

Table 5.9: The effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with the LMFL on the top 20 operational taxonomic units (OTUs) for the potential metabolically active (RNA) solid associated bacterial population at 24 and 48 hours

Genus	RF-	RF+	sed	BD-	BD+	SED	24	48	sed	RF	BD	T	RF X BD	RF X T	BD X T	RF X BD X T
Fibrobacter	32.8	35.1	3.340	34.0	33.9	2.130	32.2	35.6	2.080	0.529	0.987	0.119	0.515	0.100	0.873	0.399
OTU 3	7.37	6.98	0.980	7.85	6.50	0.827	10.23	4.12	0.817	0.717	0.115	<0.001	0.563	0.673	0.882	0.319
Paraprevotella	6.16	6.04	0.372	6.81	5.40	0.635	8.53	3.67	0.649	0.762	0.034	<0.001	0.547	0.591	0.465	0.524
OTU 5	5.54	5.85	1.640	4.86	6.53	0.898	6.03	5.36	0.863	0.858	0.064	0.450	0.684	0.703	0.603	0.599
OTU 7	5.08	5.65	1.300	4.46	6.27	0.820	5.51	5.22	0.829	0.683	0.036	0.727	0.703	0.808	0.631	0.484
OTU 19	5.98	2.60	1.230	4.81	3.78	1.210	1.49	7.09	1.231	0.052	0.402	<0.001	0.859	0.045	0.503	0.388
OTU 74	3.45	4.68	0.823	4.31	3.83	0.337	4.9	3.24	0.334	0.209	0.171	<0.001	0.969	0.250	0.567	0.399
Treponema	3.33	2.90	0.579	3.43	2.80	0.457	3.83	2.39	0.494	0.496	0.179	0.004	0.053	0.645	0.536	0.025
OTU 4	2.60	3.37	0.851	2.76	3.21	0.251	2.76	3.21	0.225	0.418	0.078	0.082	0.405	0.671	0.137	0.446
Prevotella	2.15	1.73	0.443	1.95	1.93	0.173	1.76	2.12	0.181	0.394	0.944	0.050	0.479	0.018	0.268	0.630
Butyrivibrio	1.36	1.08	0.284	1.14	1.31	0.241	0.62	1.83	0.225	0.378	0.482	<0.001	0.845	0.981	0.892	0.783
Ruminococcus	0.92	1.44	0.367	1.21	1.15	0.157	1.00	1.37	0.177	0.230	0.725	0.025	0.473	<0.001	0.518	0.520
OTU 580	0.90	1.27	0.232	1.15	1.02	0.077	1.27	0.89	0.079	0.185	0.096	<0.001	0.598	0.210	0.731	0.168

Anaeroplasma	1.05	0.87	0.205	1.09	0.84	0.258	1.21	0.72	0.251	0.435	0.342	0.068	0.887	0.258	0.416	0.468
OTU 285	1.13	0.67	0.307	0.69	1.11	0.250	0.33	1.48	0.266	0.206	0.101	<0.001	0.670	0.195	0.040	0.781
OTU 26	0.81	0.78	0.282	0.75	0.84	0.094	1.00	0.59	0.091	0.933	0.322	<0.001	0.544	0.552	0.423	0.344
OTU 87	0.78	0.64	0.058	0.79	0.64	0.107	1.06	0.37	0.103	0.081	0.161	<0.001	0.633	0.835	0.848	0.615
OTU 452	0.56	0.65	0.104	0.63	0.58	0.064	0.75	0.45	0.063	0.455	0.495	<0.001	0.816	0.114	0.938	0.645
OTU 531	0.52	0.67	0.197	0.52	0.67	0.071	0.54	0.65	0.072	0.509	0.053	0.137	0.440	0.817	0.688	0.192
OTU 138	0.67	0.41	0.182	0.42	0.67	0.141	0.13	0.95	0.143	0.227	0.088	<0.001	0.584	0.212	0.255	0.946

- Absence of the LMFL
+ Presence of the LMFL

A large proportion of the top 20 OTUs in both populations were unidentified at the genera level which is not uncommon with 16S rRNA sequencing. For example, Cheng *et al.* (2017) observed a large proportion of OTUs to be unclassified at the genera level when investigating the temporal colonisation of Rice straw possibly suggesting this to be associated with substrate. Table 5.10 displays the taxonomic classification of the unidentified OTUs as determined by the Ribosomal Database Project. Unidentified OTUs were from the phylum *Bacteroidetes* and *Firmicutes*, with the majority of OTUS belonging to the phyla *Bacteroidetes*. Genera within *Bacteroidetes* and *Firmicutes* have also been reported as the most abundant phyla within other 16 s rRNA studies such as those by (Jami & Mizrahi, 2012, Jami *et al.*, 2014, Deusch *et al.*, 2017). A large proportion of OTUs from *Bacteroidetes* were unclassified at the family level, however of the OTUs identified the families *Prevotellaceae*, *Marinilabilaceae*, *Porphyromonadaceae* and *Prevotellaceae* were present. Attempts were made to identify the unidentified OTUs further using BLAST (Morgulis *et al.*, 2008), however no further identification was found with majority of OTUs identified as uncultured rumen bacterium sequences/ partial sequences from the 16S rRNA gene.

Table 5.10: Taxonomic classification of unclassified operational taxonomic units (OTU) at the genus level present in the top 20 OTUs for the total (DNA) and potential metabolically active (RNA) solid associated bacterial population

OTU	Family	Order	Class	Phylum
3	*Prevotellaceae*	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
4	X	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
5	X	*Cytophagales*	*Cytophagia*	*Bacteroidetes*
7	X	*Cytophagales*	*Cytophagia*	*Bacteroidetes*
9	Erysipelotrichaceae	*Erysipelotrichales*	*Erysipelotrichia*	*Firmicutes*
16	X	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
19	Marinilabiliaceae	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
22	X	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
25	Lachnospiraceae	*Clostridiales*	*Clostridia*	*Firmicutes*
26	Porphyromonadaceae	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
74	X	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
87	Prevotellaceae	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
38	X	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
285	X	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
452	X	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
531	X	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*
580	x	*Bacteriodales*	*Bacteroidia*	*Bacteroidetes*

x = Unclassified

5.5.4 Experiment 1: *In-vitro* study – Predicted Metabolic Function of Bacteria

Analysis of the OTU table at the genera level was carried out to predict the potential function of the SABP within the total (DNA) and potential metabolically active (RNA) communities and to see if experimental factors had any effect on bacterial function. This was carried out by uploading the OTU table along with a FASTA file containing the OTU sequences to CowPI (Wilkinson *et al.*, 2018), which is a predictive tool as described in Chapter 2 section 2.11. Genes associated with carbohydrate and protein metabolism were specifically selected for investigation as the SABP is generally associated with the degradation of dietary substrate and it was hypothesised that the LMFL would have a stimulating effect on rumen microbiota and thus increase the degradation potential of the forage. The genes selected were those associated with, amino acid metabolism, carbohydrate digestion and absorption, carbohydrate metabolism, methane metabolism and nitrogen metabolism.

To visualise if any differences occurred between the potential function of bacteria in the total and potential metabolically active SABP principal component analysis (PCA) was conducted (Figure 5.12). PCA revealed there to be differences between the bacterial function of the total and potential metabolically active SABP which was confirmed by one-way PerMANOVA ($p=0.0001$).

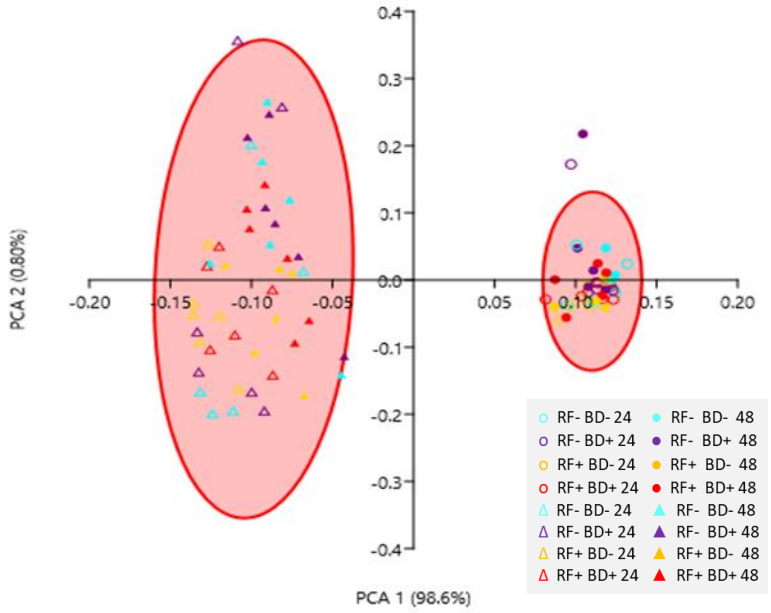


Figure 5.12: Principal component analysis (PCA) demonstrating the effect of pre-conditioning the rumen (RF) to the low moisture feed lick (LMFL), supplementation of the *in-vitro* bottle diet (BD) with the LMFL on the predicted bacterial function associated with carbohydrate and protein metabolism on the total (DNA;o) and potential metabolically active (RNA;Δ) solid associated bacterial function at 24 (non-filled shapes) and 48 (filled shapes) hours. Where – represents the absence of the LMFL and + represents the presence of the substrate. Red filled ovals represent 95% confidence ellipses for DNA and RNA.

A heat map based on fold change was created (Figure 5.13) and MANOVA calculated for the predicted gene expression of the total (DNA) and potential metabolically active (RNA) SABP. The potential metabolically active SABP had a significantly greater expression of genes associated with amino acid metabolism ($p<0.001$), carbohydrate digestion and metabolism ($p<0.001$), carbohydrate metabolism ($p<0.001$), methane metabolism ($p<0.001$) and nitrogen metabolism ($p<0.001$) in comparison with the total SABP. This suggests the up-regulation of genes associated with carbohydrate and amino acid metabolism in the potential metabolically active population which is unsurprising due to this population being associated with the transcription of genes. Moreover, it was evident that the greatest up regulation were genes associated with carbohydrate digestion and absorption.

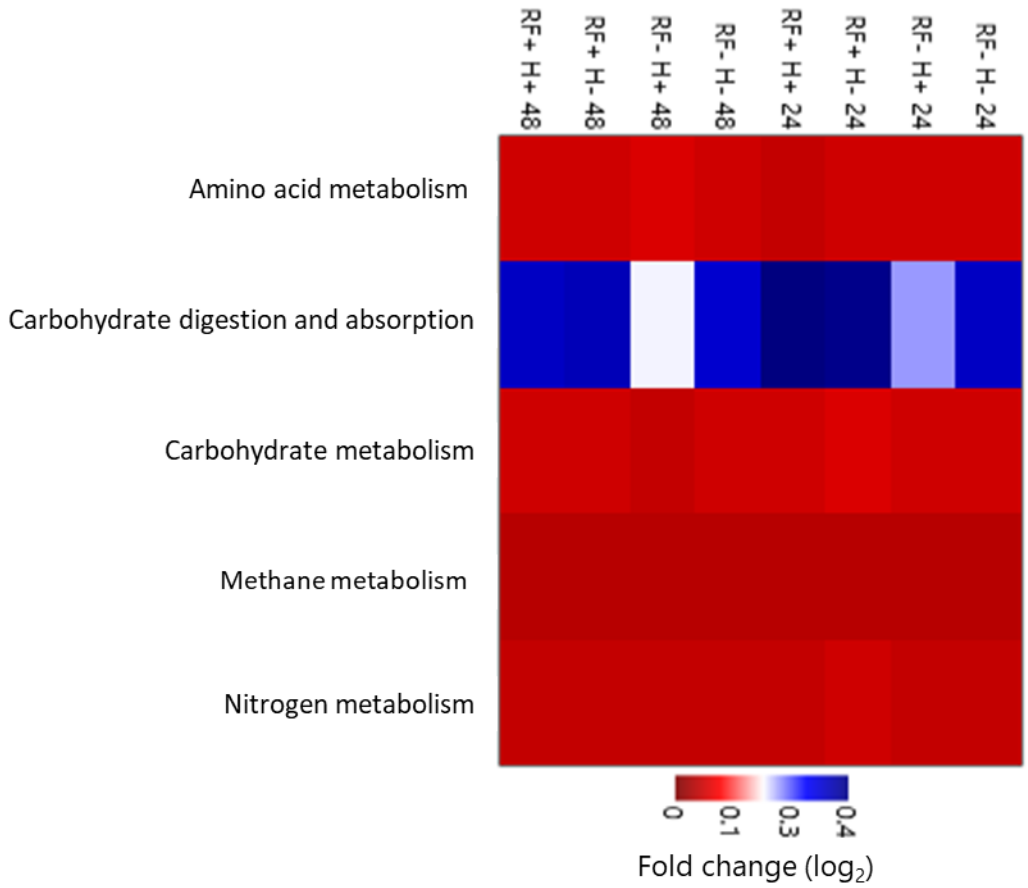


Figure 5.13: Heat map based on the fold change (log_2) between the total (DNA) and potential metabolically active (RNA) solid associated bacterial population for genes associated with carbohydrate and protein metabolism. Experimental factors involved, the pre-conditioning of the rumen (RF) to the low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with the LMFL at 24 and 48 hours. Where – represents the absence of the LMFL and + represents the presence of the LMFL.

The PCA plot (Figure 5.12) demonstrated there to be no visual differences between experimental factors in the total or potential metabolically active SABP. However, there was a visual difference between bacterial function at time points 24 and 48 hours in the potential metabolically active SABP. To investigate further PCA plots were created within populations (Figure 5.14). PCA plots revealed pre-conditioning of the rumen to the LMFL to potentially effect bacterial function in the total and potential metabolically active SABP, however this would need further statistical analysis to confirm as presented later in this section. There were no visual differences in gene function detected when the *in-vitro*

bottle diet was supplemented with the LMFL or with time in the total or potential metabolically active SABP.

Heat maps based on fold change were generated (Figure 5.15) and MANOVA calculated to determine if experimental factors had any effect on the predicted function of the total and potential metabolically active SABP. In the total SABP pre-conditioning of the rumen to the LMFL had a tendency to reduce the expression of genes associated with carbohydrate metabolism ($p=0.051$), however supplementation of the *in-vitro bottle* diet with the LMFL displayed a greater expression of genes associated with carbohydrate metabolism ($p=0.042$). Time had no effect on the expression of genes associated with amino acid metabolism, carbohydrate digestion and absorption, methane metabolism and nitrogen metabolism. There were no interactions between factors. In the potential metabolically active SABP, pre-adaptation of the rumen to the LMFL and supplementation of the *in-vitro* bottle diet with the LMFL had no effect on the predicted expression of genes associated with carbohydrate and protein metabolism. However, time had the effect of increasing the predicted expression of genes associated with carbohydrate digestion and absorption ($p=0.042$) and the effect of decreasing genes associated with carbohydrate metabolism ($p<0.001$), methane metabolism ($p<0.001$) and nitrogen metabolism ($p<0.001$). There were no significant interactions between experimental factors.

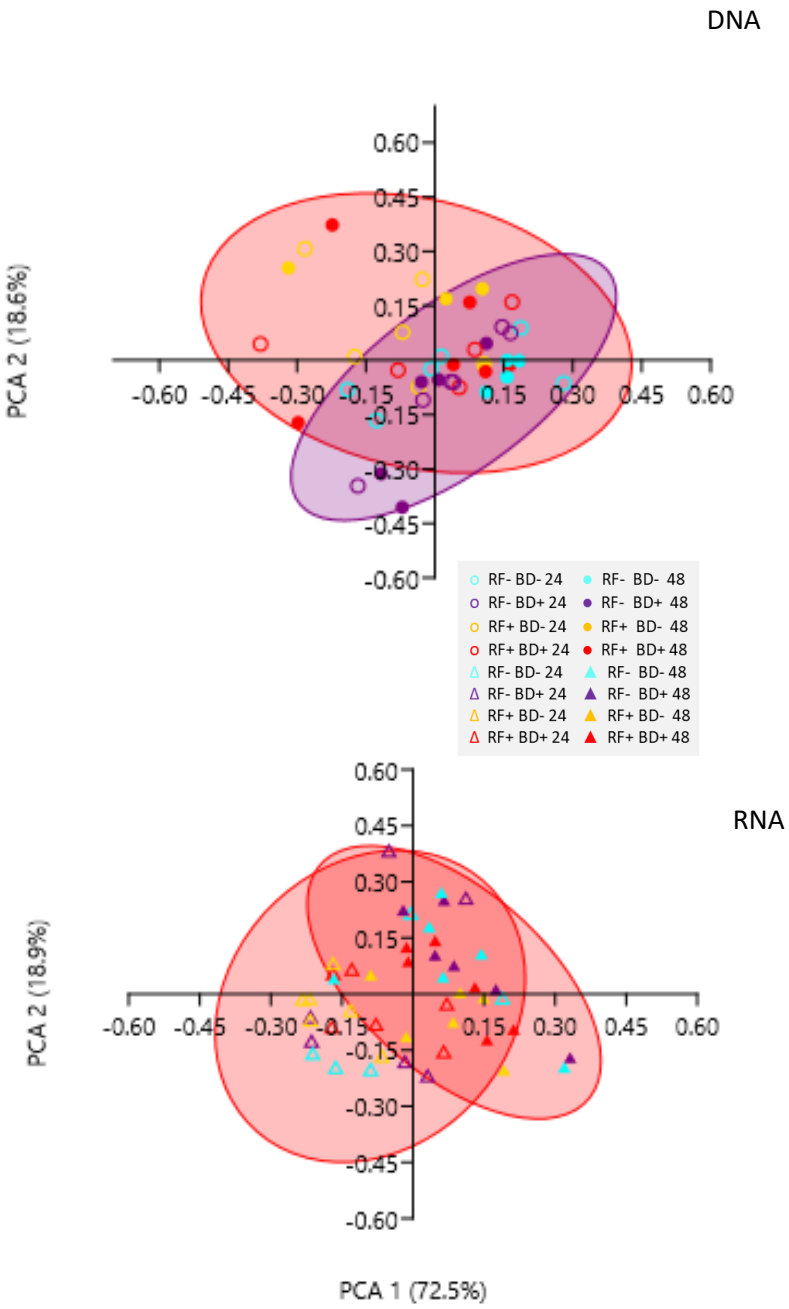


Figure 5.14: Principal component analysis (PCA) for the effect of pre-conditioning the rumen (RF) to the LMFL and supplementation of the *in-vitro* bottle diet (BD) with the LMFL on the predicted bacterial function associated with carbohydrate and protein metabolism on the total (DNA) and potential metabolically active (RNA) solid associated bacterial function at 24 (non-filled shapes) and 48 (filled shapes) hours. Where – represents the absence of the LMFL and + represents the presence of the LMFL. Filled ovals represent 95% confidence ellipses in which ellipses are RF for DNA and time for RNA.

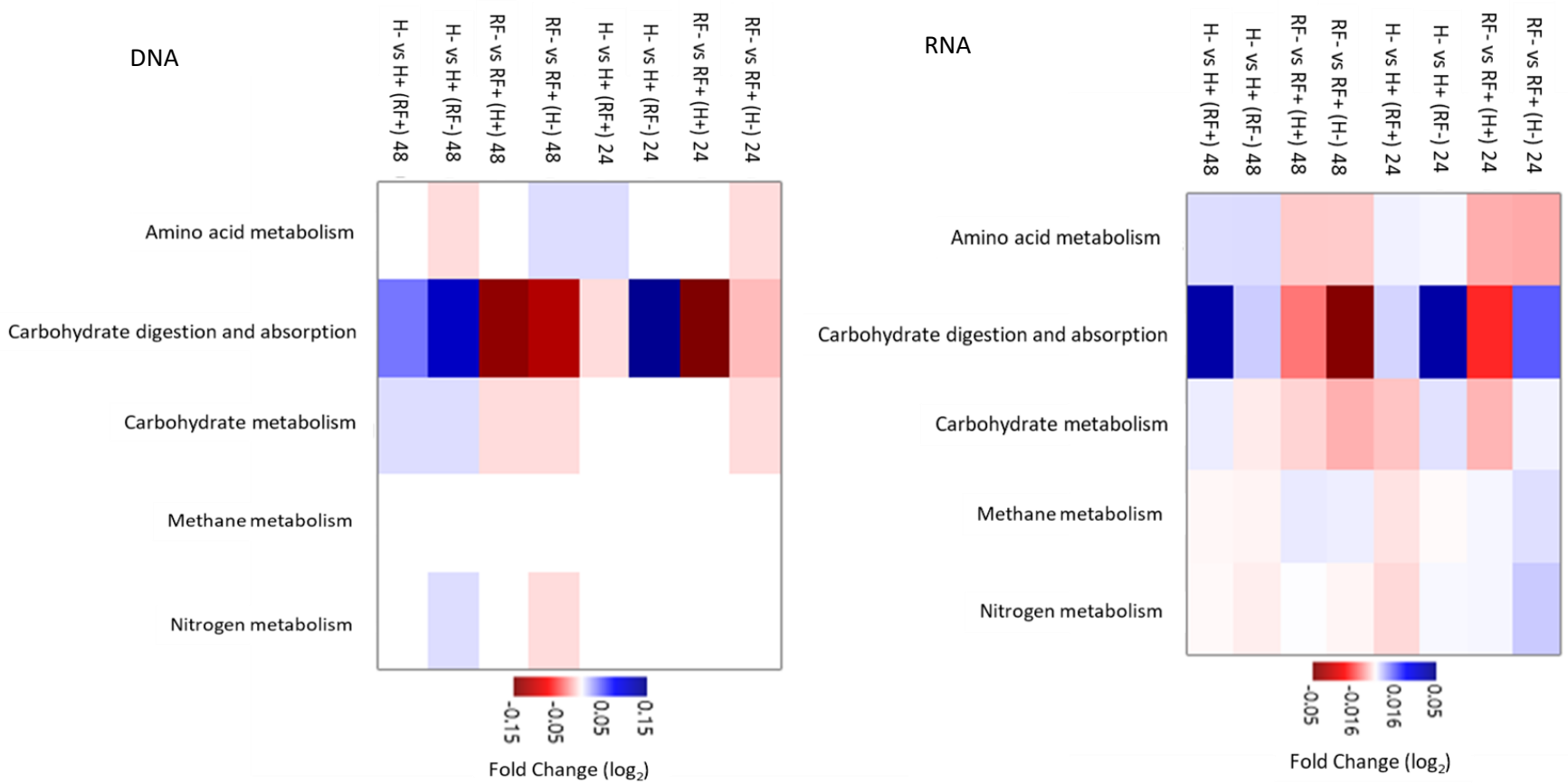


Figure 5.15: Heat map based on fold change for the effect of pre-conditioning the rumen (RF) to low moisture feed lick (LMFL) and supplementation of the *in-vitro* bottle diet (BD) with the LMFL on the predicted function of the total (DNA) and potential metabolically active (RNA) solid associated bacteria for genes associated with carbohydrate and protein metabolism at time points 24 and 48 hours. Where – represents the absence of the LMFL and + represents the presence of the LMFL.

5.5.5 Experiment 2: *In-sacco* Study

An *in-sacco* study was conducted to determine the effect of the LMFL supplementation on the rate of forage degradation within the rumen (Figure 5.16). Supplementation of the diet with free access to the LMFL demonstrated no effect on the percentage of forage dry matter that had degraded in the rumen over 48 hours. Time had a significant effect on the percentage of forage dry matter that degraded ($p<0.001$). There were no interactions between experimental factors. There was no effect of LMFL supplementation on the maximal potential for fermentation (a+b) or the rate of fermentation (c) (Table 5.11).

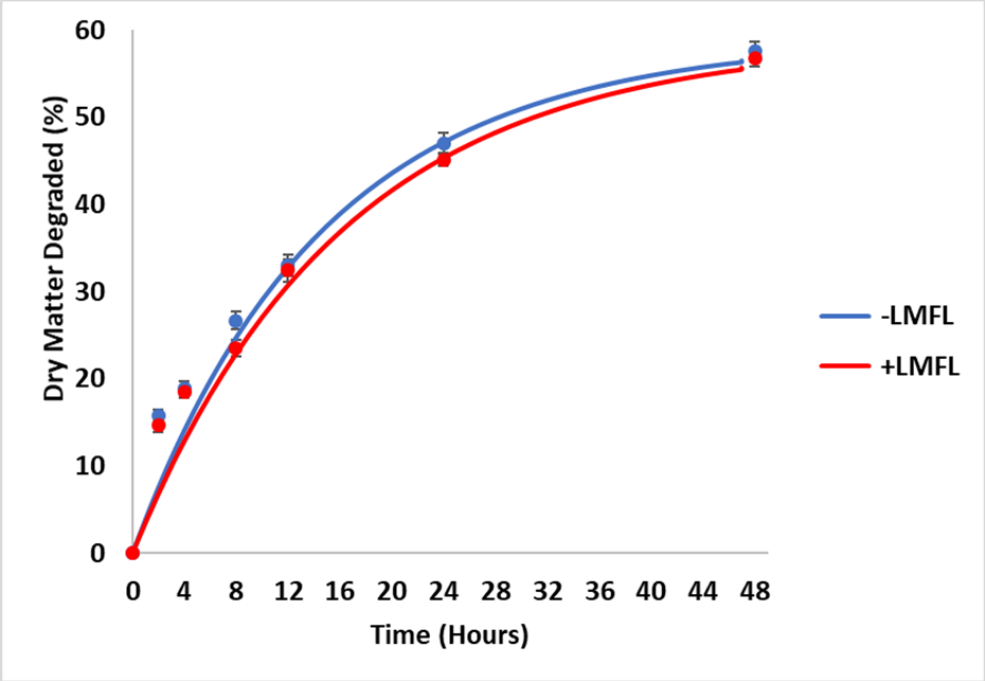


Figure 5.16: The effect of low moisture feed lick (LMFL) supplementation on the dry matter degradation of hay over 48 hours *in-sacco.* Where – represents the absence of the LMFL and + represents the presence of the LMFL. Data points represent the means of each sheep (n=6) and error bars are the standard error of the mean. Lines were calculated based on the exponential equation $Y = a + b (1e^{-ct})$ by Ørskov and McDonald (1979).

Table 5.11: The effect of low moisture feed lick (LMFL) supplementation on the kinetics of forage degradation *in-sacco* over 48 hours

	-LMFL	+LMFL	sed	p-Value
a+b	59.1	59.0	1.700	0.943
c	0.069	0.062	0.005	0.225

a+b = Maximal potential for forage degradation
c = Rate of degradation
- Absence of LMFL
+ Presence of the LMFL

5.6 Discussion

The overall aim of this chapter was to investigate the effect of pre-conditioning the rumen to the LMFL, investigating the effect of the supplement on rumen function and the SABP. To do this sheep were fed diets of Ryegrass hay and two treatments; 1) without the LMFL and 2) with the LMFL. Rumen fluid was collected from all sheep and used as part of an *in-vitro* gas production study with cross over design. The *in-vitro* gas production study also looked at the effect of LMFL supplementation of the *in-vitro* bottle diet which consisted of Ryegrass hay. Rumen function was investigated by measuring the dry matter degradation of forage and fermentation parameters, total cumulative gas volume, VFAs and ammonia. The degradation of forage was also measured in an *in-sacco* experiment which ran alongside the *in-vitro* experiment. Investigations were also made into the effect of pre-conditioning the rumen to the LMFL and supplementation of the *in-vitro* bottle diet with the LMFL on the structure and diversity of the total and potential metabolically active solid associated bacterial community. This was achieved via the co-extraction of DNA and RNA from samples, amplification of the V1/V2 hypervariable region of the 16S rRNA gene and sequencing of amplicons via Ion Torrent NGS.

There are many considerations needed when conducting NGS studies, such as; the protocol for nucleic acid extractions, amplification of the target gene, sequencing and bioinformatics analysis (Daber *et al.*, 2013). Yu & Morrison (2004) demonstrated the yield of DNA extracted from rumen digesta and faecal samples to vary when using three different extraction protocols. Likewise, Vaidya *et al.* (2018) observed different protocols for the extraction of DNA from rumen samples to result in different abundances of taxa being identified. For example, an extraction method involving a phenol dependent bead beating protocol resulted in a higher relative abundance of *Ruminococcaceae* and lower relative abundance of *Fibrobacteraceae* in comparison to the extraction protocol "Fast Spin DNA kit for soil". Moreover, Henderson *et al.* (2013) observed differences to occur in the microbial community structure of the same rumen sample when 15 different extraction protocols were tested, cautioning the comparison of studies using different nucleic acid extraction protocols. The amplification process

has been identified to implement a certain degree of bias into studies as a result of the primers used, different hypervariable regions of the gene sequence targeted and different cycling conditions during amplification. For example, Li *et al.* (2016) suggests primer design for known/ conserved sequences to exclude the opportunity to sequence novel phylotypes. Likewise, in a review study Huws *et al.* (2018) demonstrated the importance of primer selection for targeting the hypervariable region of the 16s rRNA gene, in which primers targeting the V3 region demonstrated the ability to amplify chloroplast/cyanobacteria, certain 16S rRNA archaeal and certain 18S rRNA protozoal sequences.

5.6.1 Rumen Function

It was hypothesised that pre-conditioning the rumen to the LMFL and supplementation of the *in-vitro* bottle diet would increase the degradation and fermentation potential of forage due to an increase in microbial activity as a result of the provision of the supplement to the diet.

Pre-conditioning the rumen to the LMFL and supplementation of the *in-vitro* bottle diet with the LMFL had no effect on the dry matter degradation of forage over 48 hours (Experiment 1). In the *in-vitro* experiment small quantities of substrate were used which demonstrated the ability to increase the margin for technical error occurring when aiming to recover and re-weigh residual substrate as experienced in Chapter 4. Therefore, the dry matter degradation of forage was also measured *in-sacco* study (Experiment 2). The *in-sacco* study demonstrated there to be no effect of LMFL supplementation on the percentage of forage dry matter that had degraded over 48 hours. These results disagree with those in Chapter 4 which used the same LMFL and the same type of forage as used in this chapter. The results of Chapter 4 suggested a trend for the LMFL to have the effect of increasing the percentage of forage dry matter that had degraded over 24 hours. However, statistically there was no significant difference, thought to be attributed to technical error in the recovery of residual substrate. The results of this Chapter are also in disagreement with *an in-vitro* study with slaughter cattle by Chaudhry (2008) who observed LMFL supplementation to have an increasing effect on the dry matter degradation of barley straw and grass nuts over 72 hours. However Leupp *et al.* (2005) observed steers fed Switchgrass hay supplemented with a LMFL to have no effect on the rate of dry matter (DM), neutral detergent fibre or acid detergent fibre degradation over 98 hours *in-sacco*.

The effect of LMFL supplementation on the total tract digestibility of forages has been measured in studies in cattle *in-vivo* as previously discussed in Chapter 4 section 4.6.1. These studies demonstrated the supplementation of steers consuming the LMFL supplement to have an increasing effect on the DM, OM, NDF and N digestibility of poor-quality tropical forages (*ad-libitum*) and in turn an increasing effect on voluntary DM, OM, NDF and N digestibility forage intakes (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Leupp *et al.*, 2005). Likewise, Titgemeyer *et al.* (2004)

demonstrated heifers consuming Prairie grass hay supplemented with a low (CP, 144 g/ kg DM) or high (CP, 275 g/ kg DM) protein LMFL to have the effect of increasing the dry matter digestibility of the diet, however when the forage was supplemented with 1.96 kg/ day of Alfalfa hay the LMFLs had no effect on the digestibility of the diet. This is perhaps indicative of the LMFL having no effect on metabolism when the diet is nutritionally sufficient for maintenance and production demand. Likewise, in an additional study by Titgemeyer *et al.* (2004), LMFL supplementation demonstrated no effect on the voluntary forage DM, OM, NDF or N intakes or the DM, OM and NDF digestibility of the diet when steers were fed diets of either Alfalfa hay (*ad-libitum*), Brome hay (*ad-libitum*) or Brome hay plus 1.93 kg/ day of Alfalfa hay.

The chemical analysis of Ryegrass hay in this Chapter had a greater NDF (773 g/ kg DM) content in comparison with the forages fed by Greenwood *et al.* (2000) (Prairie hay, 694 g/ kg DM), Leupp *et al.* (2005) (Switchgrass,747 g/ kg), Löest *et al.* (2001) (Prairie hay, 695 g/ kg NDF), Titgemeyer *et al.* (2004) (Prairie hay, 730 g/kg; Alfalfa hay, 513-600 g/ kg; Brome hay, 729 g/ kg), Chaudhry (2008) (Barley straw, 859 g/ kg) suggesting the forage in this study to have a higher hemicellulose, cellulose and lignin content. However the CP content of the Ryegrass hay (80.3 g/kg DM) in this study was much higher in comparison with forages fed by, Greenwood *et al.* (2000) (Prairie hay, 59 g/ kg DM), Leupp *et al.* (2005) (Switchgrass,59 g/ kg), Löest *et al.* (2001) (Prairie hay, 55 g/ kg NDF) and Chaudhry (2008) (Barley straw, 32 g/ kg) whom all observed an increasing effect of LMFL supplementation on the digestibility of the diet. However the crude protein content of the forage in this study was lower in comparison with Titgemeyer *et al.* (2004) (Alfalfa hay 186-191 g/kg; Brome hay, 84 g/ kg) whom observed LMFL supplementation to have no effect on the digestibility of forages. Titgemeyer *et al.* (2004) hypothesised LMFL supplementation to only effect the digestibility of forage when the rumen degradable protein fraction of the diet was limiting. This is perhaps indicative of why LMFL supplementation in this study did not affect the degradation of the Ryegrass hay. In addition, the sheep in this study were geriatric, had long reached maturity and only had a nutritional requirement for maintenance level, therefore it was more than likely the forage provided in this study was sufficient in rumen degradable protein. Likewise, the buffer component of the inoculum did not contain an additional protein source (cysteine hydrochloric acid, trypticase peptone) as is the case with alternative versions of the buffer, for example that used by Asanuma *et al.* (1999). The buffer did contain ammonium hydrogen carbonate as a means of maintaining the pH which may have contributed to available N in the inoculum for metabolism by rumen microbiota. However, Chaudhry (2008) observed LMFL supplementation to have an increasing effect on the dry matter degradation of grass nuts which were of a high nutritional quality in comparison with all forages investigated (606

g/kg NDF; 168 g/kg CP) in the literature. However, in this study forage was ground and therefore may have increased the degradation potential of forage through increased microbial access.

Fermentation parameters; the volume of gas produced, VFAs and ammonia were measured as a potential indicator of fermentation as previously conducted in Chapter 4 and discussed in section 4.6.2. Pre-conditioning the rumen to the LMFL had no effect on the cumulative volume of gas produced over 48 hours or the molar concentrations of total VFAs and the major individual VFAs; acetate, propionate and butyrate. However, supplementation of the *in-vitro* bottle diet with the LMFL had the effect of increasing the cumulative volume of gas produced over 48 hours, the molar concentrations of total VFAs and the individual VFAs, acetate, propionate, butyrate and the branched chain VFAs. These observations agree with those made in Chapter 4 in which LMFL supplementation in the absence and presence of dietary substrate had the effect of enhancing fermentation over 24 hours. However, the results of this chapter differ in places from results in Chapter 4. For example, LMFL supplementation demonstrated the effect of reducing the ratio of acetate to propionate (A:P), whereas in this study LMFL supplementation of the *in-vitro* bottle diet had the effect of increasing A:P. Likewise, LMFL supplementation demonstrated no effect on the molar concentration of the branched chain VFAs whereas in this chapter LMFL had an increasing effect on the molar concentration of the branched chain VFAs. These results demonstrate the pre-conditioning of the rumen to have no effect on rumen fermentation. However, when the supplement is provided to the diet as a flat rate dose the supplement has the effect of enhancing fermentation. This suggests the LMFL to have an additive effect in the rumen, where the product supplies a readily available source of fermentable carbohydrate which can be utilised as a substrate by rumen microbiota, most likely the liquid associated population. Moreover, these results are indicative of the LMFL having a localised effect on rumen fermentation rather than any long-term lasting effect.

Studies investigating the effect of LMFL supplementation on rumen function are limiting, however fermentation parameters have been measured in whole animal trials in cattle. Leupp *et al.* (2005) observed a beet molasses based LMFL to have no effect on the molar concentrations of total VFAs, or individual VFAs; acetate, propionate and butyrate. This agrees with the observation made in this chapter in which the pre-conditioning of the rumen demonstrated no effect on rumen fermentation. Likewise, study by Greenwood *et al.* (2000) demonstrated steers fed Prairie hay supplemented with a cane molasses based LMFL to have no effect on the molar concentration of total VFAs or individual VFAs; acetate, propionate or butyrate, however the supplement had the effect of decreasing the molar concentrations of the branched chain VFAs. In the same study a beet molasses based LMFL and a concentrated separator by-product (from the sugar refining industry) based LMFL were fed and demonstrated the effect of increasing the molar concentrations of total VFAs and individual VFAs;

acetate, butyrate and propionate and the effect of decreasing the molar concentration of the branched chain VFAs. These results are similar to observations made in this chapter in which supplementation of the *in-vitro* bottle diet with the LMFL had an enhancing effect on fermentation.

It must be remembered that LMFLs are a type of supplement and there are many different products with different dietary ingredients and specifications and thus may influence fermentation and the extent of fermentation. For example, the LMFL used in this thesis was a cane molasses based LMFL and had a total sugar content of 380 g/ kg DM and a crude protein content of 120 g/ kg DM as described in Chapter 2 section 2.2.1. Whereas, the sugar content of the cane molasses LMFL fed by Greenwood *et al.* (2000) was 329 g/ kg DM and the crude protein content 307 g/ kg DM, the sugar content of the beet molasses LMFL was 348 g/ kg DM and the CP content 316 g/ kg DM and the sugar content of the concentrated separator by-product block 159 g/ kg DM and the crude protein content 299 g/ kg DM. Likewise, the crude protein content of the LMFLs fed by Leupp *et al.* (2005) were on average 362 g/ kg DM, it was not disclosed what the sugar content of the LMFLs were.

Pre-conditioning the rumen to the LMFL and supplementation of the *in-vitro* bottle diet with the LMFL had no effect on the molar concentration of ammonia. This disagrees with observations by Leupp *et al.* (2005) and Greenwood *et al.* (2000) who observed the molar concentrations of ammonia to increase with LMFL supplementation. As described above the crude protein content of the LMFLs in studies by Greenwood *et al.* (2000) ranged from 299 - 316 g/ kg DM and from 311 - 405 g/ kg DM by Leupp *et al.* (2005) which is much greater in comparison with the LMFL used in this study which contained 120 g/ kg DM as described in Chapter 2 section 2.2.1. Likewise, there was a higher content of non-protein nitrogen within the LMFL by Greenwood *et al.* (2000) and Leupp *et al.* (2005).

5.6.2 Low Moisture Feed Lick Supplementation and the Solid Associated Bacterial Population

Bacteria are recognised as the most abundant and diverse kingdom of microbiota within the rumen, responsible for the degradation and fermentation of plant material (Deusch *et al.*, 2017). The bacterial community associated with feed particles are estimated to comprise of 75% of the total microbial population (Wang *et al.*, 2017). Previous research has demonstrated LMFL supplementation to increase the digestibility of poor quality tropical forages fed to cattle (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Leupp *et al.*, 2005). Likewise, the results from Chapter 4 demonstrated a trend for LMFL supplementation to have the effect of increasing the dry matter degradation of forage *in-vitro*, a similar observation to that made by Chaudhry (2008). Therefore, it was hypothesised that the LMFL would affect the structure and diversity of the SABP thus resulting in an increase in the degradation potential of forage.

The effect of LMFL supplementation on the structure and diversity of the SABP was conducted via NGS technology. Nucleotides DNA and RNA were co-extracted from residual solid samples to investigate the total and potential metabolically active SABP. To do this the bacterial 16S rRNA gene was targeted and sequenced using specific primers. This gene is approximately 600 base pairs in length and contains nine hypervariable regions (V1-V9) of varying conservation (Bukin et al 2019). To knowledge there have been no other molecular studies investigating the effect of LMFL supplementation on the rumen microbiome.

Pre-conditioning of the rumen to the LMFL had no effect on the structure of the SABP in the total or potential metabolically active SABP, however within each time point LMFL supplementation demonstrated a tendency to effect the structure of the SABP in both the total and potential metabolically active population, with more of an effect in the potential metabolically active SABP. This suggests pre-conditioning of the rumen to the LMFL to have a minor effect on the structure of the SABP maybe because of nutrient supply or differential RNA expression. Likewise, there was no effect of LMFL supplementation on the metabolic activity of the SABP in which the degradation of forage and rumen fermentation was unaffected as discussed in section 5.6.1, further suggesting this population to be unaffected by the supplement. There was no effect of supplementation of the *in-vitro* bottle diet with the LMFL on the structure or diversity of the total or potential metabolically active SABP. Likewise, there was no effect of supplementation within time points. Pre-conditioning of the rumen to the LMFL and supplementation of the *in-vitro* bottle diet with the LMFL had no effect on the alpha diversity of the total or potential metabolically active SABP suggesting bacterial diversity and abundance of the different species to be similar across populations. It must be noted that a limitation of this study was that rumen microbiota were collected following the filtering of rumen digesta through muslin, therefore it was more likely that the majority of microbiota were heavily associated with the liquid associated population.

This chapter also investigated the potential functionality of the SABP using the predictive tool CowPI (Wilkinson *et al.*, 2018) for genes associated with carbohydrate and protein metabolism. In the total SABP supplementation pre-conditioning of the rumen to the LMFL had a tendency to decrease the expression of genes associated with carbohydrate metabolism. However, supplementation of the *in-vitro* bottle diet with the LMFL had an increasing effect on the potential expression of genes associated with carbohydrate metabolism. There was no effect of supplementation on other genes associated with carbohydrate or protein metabolism. In the potential metabolically active SABP there was no effect of LMFL supplementation on the potential function of genes associated with carbohydrate and protein metabolism. These results perhaps suggest that the LMFL supplement has little effect on the

composition and functionality of the SAP and is perhaps indicative of why supplementation had no effect on the degradation of forage in this chapter.

The results of this chapter were indicative of LMFL supplementation having an enhancing effect on fermentation. The SABP are not readily involved in the fermentation of readily fermentable carbohydrates which produce the fermentation products; gas (CO_2, CH_4) and VFAs, instead the population is involved in the degradation of substrate (McAllister *et al.*, 1994, Belanche *et al.*, 2017). Therefore, if anything the LMFL is likely to affect the liquid associated population. Further research should be required on residual liquid samples from this study to confirm this.

5.6.3 Time and the Solid Associated Bacterial Population

Bottles were subjected to sacrificial harvest 24 and 48 hours post inoculation. Differences resided between the structure of the SABP in the total and potential metabolically active populations at 24 and 48 hours. Time demonstrated no effect on the alpha diversity of both populations when using the inverse Simpson index, however in the Shannon index time had the effect of increasing the diversity of the SABP in the total population and a tendency to increase the diversity in the potential metabolically active population. In the total SABP time had an increasing effect on phyla, *Firmicutes*, other phyla and unclassified phyla and a decreasing effect on *Spirochaetes.* Whereas time had an increasing effect on *Firmicutes, Proteobacteria* and a decreasing effect on *Spirochaetes* in the potential metabolically active bacterial SABP. Time also had the greatest effect out of all factors measured on the top 20 OTUs identified in the total and potential metabolically active SABP.

Time has been demonstrated to have a significant effect on the structure and diversity of the SABP. Huws *et al.* (2016) demonstrated microbiota colonising forage to be a biphasic process with the epiphytic plant community being replaced with a primary colonising community and then shifting to a secondary colonising community. Similar observation was made by Belanche *et al.* (2017), although the concept of a tertiary colonising community was introduced in which the majority of microbiota within this population were renowned for having fibrolytic activity. Edwards *et al.* (2008) demonstrated the initial colonisation of fresh Perennial Ryegrass to occur within 5-30 minutes of exposure to the rumen. Using the same substrate of Perennial Ryegrass Huws *et al.* (2013) and Mayorga *et al.* (2016) both demonstrated primary colonisation to occur within 0-2 hours before shifting to a secondary colonising community post 4 hours of exposure to rumen microbiota. Huws *et al.* (2014) demonstrated forage chemical and physical composition to affect the time frame in which the events of colonisation occur. A similar observation was made by Elliott *et al.* (2018) who demonstrated the primary colonisation of fresh Perennial Ryegrass to take up to 4 hours but the primary colonisation of forages Birdsfoot Trefoil and Red Clover to take up to 6 hours before

replacement by a secondary colonising community. Using conserved forages, Piao *et al.* (2014) and (Cheng *et al.*, 2017) observed the primary colonisation of Rice straw, Alfalfa hay and Switchgrass hay to take up to 6 hours before replacement by the secondary colonising community post 6 hours. Belanche *et al.* (2017) investigated the colonisation of fresh Ryegrass and Ryegrass hay, demonstrating the events of primary colonisation to occur within 0-2 hours before replacement by a secondary colonising community at 4-8 hours. The events of colonisation were observed to take longer for the conserved forage in comparison with the fresh forage with another community shift being observed post 8 hours, described as a tertiary colonising community. This tertiary community was simplex and slower to establish in comparison with the other communities identified. In this chapter the events of colonisation were investigated at 24 and 48 hours therefore the community identified is most likely to be the tertiary colonising community. Moreover, majority of microbiota identified were from the phyla *Fibrobacteres* and *Bacteroidetes* which are renowned for being high in relative abundance in the gastrointestinal tract of herbivores and have fibrolytic activity (Jami & Mizrahi, 2012, Jami *et al.*, 2014, Deusch *et al.*, 2017).

5.6.4 Total vs Potential Metabolically Active Solid Associated Bacterial Population

There are many considerations that need to be made when conducting 16s rRNA studies for the identification of bacterial communities. For example, differences in protocols for nucleic acid extraction, primer design, targeting of different hypervariable regions of the 16S rRNA gene, amplification cycles, the use of different sequencing machines and bioinformatics pipelines (Huws *et al.*, 2018) as discussed in section 5.1.

The co-extraction of DNA and RNA using the protocol by Griffiths *et al.* (2000) allowed for comparison of the total and potential metabolically active SAP thereby reducing any bias that may be introduced by extracting DNA and RNA using two different protocols. Post quality filtering 6888 and 6672 sequences resided for DNA and RNA respectively and were clustered into 464 and 388 different OTUs respectively. This is relatively low in comparison with studies using low quality forages such as those by Belanche *et al.* (2016) and Belanche *et al.* (2017) who looked at the microbial colonisation of Ryegrass hay and Piao *et al.* (2014), Liu *et al.* (2016), Cheng *et al.* (2017) whom investigated the microbial colonisation of rice straw, alfalfa hay and air dried Switchgrass hay. Reasons for differences may be a result of experimental design. In this chapter time points 24 and 48 hours were selected which are relatively late time points regarding colonisation studies. Likewise, the initial dietary substrate (0.8g DM) was small and the recovery of residual substrate even smaller thereby limiting the quantity and opportunity for nucleotide extraction.

Firmicutes and *Bacteroidetes* had the greatest number of OTUs present within phyla across the total and potential metabolically active population. This is in agreement with Mi *et al.* (2018) whom also observed *Firmicutes* and *Bacteroidetes* to have the largest number of OTUs within phyla when DNA was extracted from rumen samples obtained from sheep. *Fibrobacteres*, *Bacteroidetes* and *Firmicutes* had the highest relative abundances of all phyla. *Fibrobacteres, Bacteroidetes* and *Firmicutes* have all been identified to have fibrolytic activity in the rumen with *Fibrobacteres* capable of producing and secreting cellulases for the degradation of cellulose and *Bacteroidetes* capable of having saccharolytic activity degrading plant polysaccharides aside from cellulose (Naas *et al.*, 2014, Bi *et al.*, 2018). The most abundant phyla across the total SABP extracts was *Fibrobacteres (31%)* followed by *Bacteroidetes* (45%) and *Firmicutes* (14%). This observation disagrees with Wang *et al.* (2017) who demonstrated *Firmicutes* (52%), *Bacteroidetes* (32%) and *Proteobacteria* (7.5%) to be the dominant phyla found across the digestive tract of Han sheep fed a diet of hay and a pre-mix following DNA extraction. Likewise Jami & Mizrahi (2012) observed *Bacteroidetes* (50%) and *Firmicutes (43%)* to be the dominant phyla identified in rumen digesta samples obtained from crossbred beef steers consuming high-energy finishing diet following DNA extraction. The most abundant phyla across the potential metabolically active SABP extracts were *Bacteroidetes* (52%), followed by *Fibrobacteres* (34%) and *Firmicutes* (9%). This disagrees with Belanche *et al.* (2017) who observed *Firmicutes* (69%) and *Bacteroidetes* (26%) to be the most dominant phyla in a Rusitec study using rumen fluid from cows investigating the effect of fresh Ryegrass and Ryegrass hay supplemented with vitamin E following RNA extraction. Differences may be a result of the diet and experimental conditions for example *in-vitro* vs *in-sacco* systems. Likewise, within *in-vitro* systems experimental conditions such as ratio of rumen fluid to buffer, type of buffer, volume of inoculum and substrate quantity may also be contributing factors. As discussed in section 5.6.3 the microbial population was most likely to be the tertiary community therefore time is another likely factor influencing the results observed.

Throughout this chapter it was evident that differences occurred in the structure of the total and potential metabolically active SABP reflected in differences in the relative abundance of microbiota at different phylogenetic levels. The potential metabolically active SABP had a higher relative abundance of *Bacteroidetes* in comparison with the total SABP. However, had a lower relative abundance of *Fibrobacteres* and *Firmicutes, Tenericutes* and *Spirochaetes.* Likewise, there were differences in the top 20 OTUs identified at the genera level within each population. However, there was no difference in the alpha diversity between nucleic acid extracts. It is not surprising that there were differences between the two populations as the total population describes all microbiota whether they are metabolically active, growing, dormant or deceased, whilst the potential metabolically active population describes microbiota that are participating in transcription (Popova *et al.*, 2010). However,

within the population that is “active” and undergoing the transcription of genes it can’t be certain that microbiota are actually active in metabolism as some microbiota can undergo transcription but be dormant (Blazewicz *et al.*, 2013).

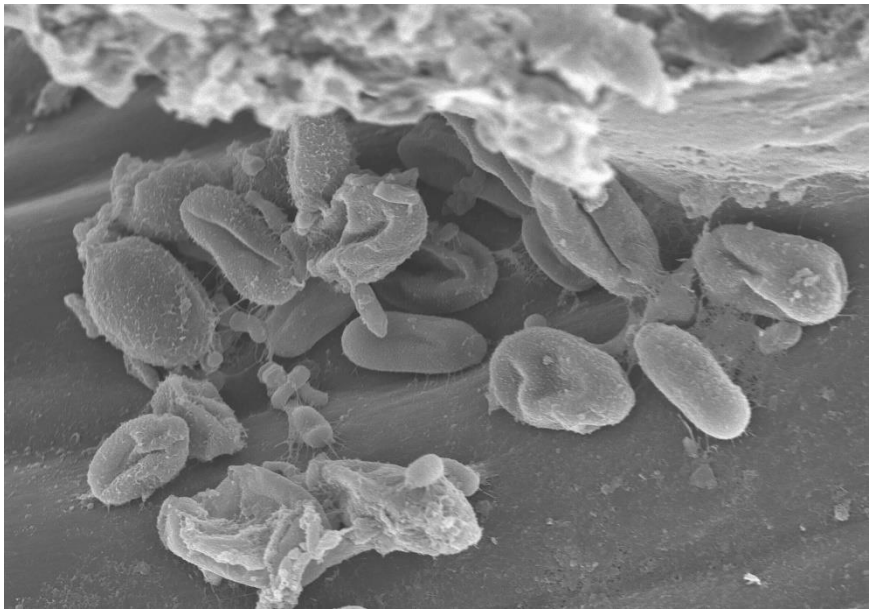
The use of 16s rRNA NGS is a good technique for the identification of microbiota present within samples, however it does not give indication as to their role/function within the rumen. Using the predictive tool CowPI (Wilkinson *et al.*, 2018) bacterial function within the total and potential metabolically active population was predicted for genes associated with carbohydrate and protein metabolism. Fold change demonstrated the expression of genes within the potential metabolically active SABP to be upregulated in comparison with the total SABP. This demonstrates the importance of using samples derived from RNA in studies when investigating microbial metabolism. Likewise, investigating the total population may mask genera of importance in metabolism due to the capacity of the total vs potential metabolically active population.

5.7 Conclusions

In conclusion, pre-conditioning the rumen of mature non-productive sheep with the LMFL supplement had no effect on the degradation of forage and rumen fermentation. Moreover, pre-conditioning of the rumen to the LMFL had little effect on the structure, diversity and function of the SABP. Supplementation of the *in-vitro* bottle diet had no effect on the degradation of forage within the rumen, however supplementation had the effect of enhancing rumen fermentation. Likewise, the LMFL had little effect on the structure, diversity or function of the SABP.

These results suggest that a flat rate dose of the LMFL has a stimulating effect on the metabolic activity of rumen microbiota, portrayed by an increase in fermentation products. This is perhaps a result of the additional dietary substrate (carbohydrate) the LMFL provides to the diet which is readily fermented by microbiota. Moreover, such microbiota are likely to be those of the liquid associated population and hence why the LMFL in this study demonstrated no effect on the structure or diversity of the SABP. However, further research would be required to confirm this. Likewise, further research is required to determine the effect of LMFL supplementation on the structure and diversity of other kingdoms of microbiota such as methanogens, anaerobic fungi and protozoa. Moreover, these results highlight the use of the LMFL in the long-term supplement rather than the short-term due to the pre-conditioning of the rumen to the LMFL having no effect on metabolism. This Chapter also demonstrates the importance of using nucleic acids derived from RNA in NGS studies for targeting the population of microbiota that are potentially active and involved in metabolism of dietary substrates.

Chapter 6: Effect of Low Moisture Feed Lick Supplementation on the Appetite and Metabolism of Sheep *in-vivo*



6.1 Introduction

Nutrition is an integral factor in animal production systems with significant contribution to animal health, performance and productivity. Forage is the most economical feed resource available for ruminants (AHDB, 2016), with the majority of grazing in the UK comprised of swards of Perennial Ryegrass mixed with legumes such as Red and White clover (Kingston-Smith *et al.*, 2013). The majority of sheep production systems in the UK are reliant on extensive grazing. However the nutritional quality and availability (Figure 6.1) of grazing is often inconsistent throughout the grazing season and at times insufficient to meet the nutritional requirements for production (growth) and at physiologically demanding times (gestation and lactation), especially for animals of high genetic merit. To compensate, it is popular to provide supplementary nutrition.

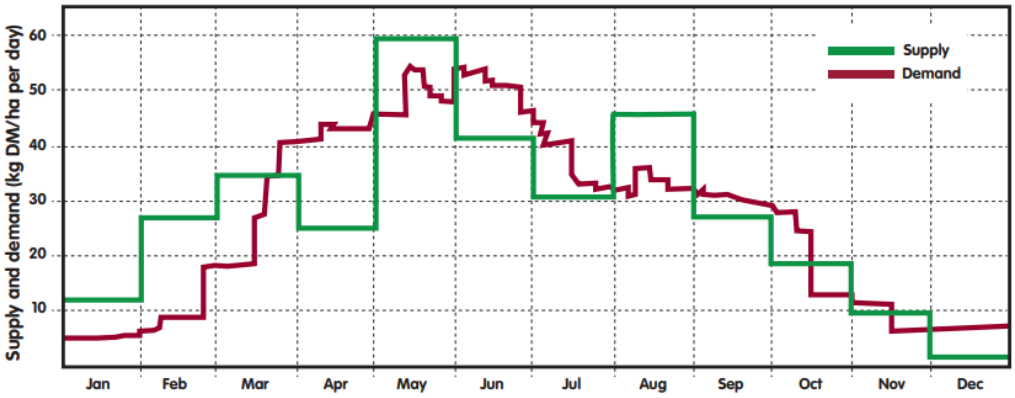


Figure 6.1: Typical annual supply and demand of grazing in the UK (AHDB, 2016).

Low moisture feed licks (LMFL) are a popular method of supplementation, providing an additional source of energy, protein, vitamins and minerals to the diet. Licks are placed out at pasture for self-regulatory consumption requiring little labour expenditure in comparison with traditional methods of supplementation such as feeding concentrates. Previous metabolism studies in cattle have demonstrated LMFL supplementation to have a complementary effect on the diet, increasing the digestibility of poor quality tropical forages and stimulating voluntary forage intakes in comparison with control animals (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Leupp *et al.*, 2005). LMFL supplementation has also demonstrated positive effects on animal performance in comparison with control animals in terms of increased live weight gain (Hart & Newbold, 2015) and improved nutritional status of animals in terms of body condition and blood metabolic status (Cabiddu *et al.*, 2014), suggesting supplementation to help improve production efficiency. However, there is limited research on the effect of LMFL supplementation on the appetite and metabolism of sheep. Previous studies in this thesis have involved the use of the *in-vitro* gas production technique. This

technique relies on a closed system and does not take into account the "animal effect" and the impact of factors such as appetite, mastication, saliva production, animal metabolism and total tract passage rate. Therefore, this study is a follow up study to Chapters 4 and 5 investigating the effect of LMFL supplementation on the appetite and metabolism of sheep *in-vivo*.

6.2 Chapter Aims

The aim of this chapter was to investigate the effect of LMFL supplementation on the appetite and metabolism of sheep *in-vivo*. The *in-vivo* study in this chapter comprised three parts, 1) Appetite study; 2) Rumen study; 3) Metabolism study. The appetite study focuses on feeding behaviour investigating forage intake, water intake and live weight. The rumen study focuses on the temporal effects of LMFL supplementation in the rumen, investigating the degradation of forage using the *in-sacco* technique and fermentation parameters. The metabolism study investigates the effect of LMFL supplementation on the digestibility of the forage-based diet and enteric methane emissions.

6.2.1 Hypothesis

The hypotheses of this chapter are detailed below:

H_1: LMFL supplementation will have an increasing effect on appetite where voluntary forage intake and live weight will increase

H_2: Due to the dehydrated nature of the LMFL, LMFL supplementation will have the effect of increasing water consumption

H_3: LMFL supplementation will have the effect of increasing the dry matter degradation of forage in-sacco

H_4: LMFL supplementation will have the effect of enhancing rumen fermentation, resulting in greater concentrations of fermentation products; VFAs and ammonia

H_5: LMFL supplementation will have a positive effect on metabolism, increasing the digestibility of nutrients from forage

6.3 Materials and Methods

This experiment was reviewed and conducted with ethical compliance in accordance with the University's guidelines and home office legislation as described in Chapter 2 section 2.1.

6.3.1 Experimental Design, Animals and Treatments

Experimental design consisted of a 2 x 3 replicated Latin Square (Table 6.1) with 21 day periods. Days 0-14 were allocated to adaptation to diet and days 15-21 allocated for sampling (Table 6.2). For practicality reasons and equipment availability, the second group began the experiment 7 days after the first group and there was a 1 day wash out period after each group had finished a period.

Table 6.1: Metabolism study experimental design

Square 1: Group A			
		Period 1 (Column)	Period 2 (Column)
Sheep (Row)	Sheep 1	-LMFL	+LMFL
	Sheep 2	-LMFL	+LMFL
	Sheep 3	+LMFL	-LMFL

Square 2: Group B			
		Period 1 (Column)	Period 2 (Column)
Sheep (Row)	Sheep 4	-LMFL	+LMFL
	Sheep 5	-LMFL	+LMFL
	Sheep 6	+LMFL	-LMFL

LMFL = Low moisture feed lick
-LMFL = control diet (hay -LMFL)
+LMFL = treatment diet (hay +LMFL)

The trial took place at the CIEL (Centre of Innovation and Excellence in Livestock) facility on Aberystwyth University's farm at Plas Gogerddan. Six Aberdale x Texel sheep (date of birth 2012; 2 male, 4 female; 87.3 kg ± 7.6 kg) fitted with rumen cannulas (Bar Diamond, Parma Idaho USA) were split into two groups of three, A and B, balanced for gender and live weight. Sheep were housed indoors in individual pens and bedded on wood shavings with free access to clean fresh water and a salt lick (Baby Pure, Rockies, Winsford, UK).

Table 6.2: Metabolism study experimental schedule

	Day of period													
	1-9	10	11	12	13	14	15	16	17	18	19	20	21	22
	Period													
Adaptation period														
Sampling period														
Clean out period														
	Sampling/ measures													
Feed intake, water intake, live weight														
Rumen fluid collections														
In-sacco incubations														
Metabolism – digestibility, N-balance														
Enteric emissions methane measured														

Sheep were fed ryegrass hay *ad libitum* (2.2% LW) and 100 g of dried unmolassed sugar beet shreds (Trident, AB Agri, Peterborough, UK) as an incentive to enter weigh crates (as described in section 6.3.2), further details on diet delivery are discussed in Chapter 2 section 2.4.1. Groups were randomly allocated to one of two experimental treatments, 1) Control (-LMFL); no supplement; 2) Treatment (+LMFL); low moisture feed lick supplement. The LMFL under investigation was Crystalyx® Extra High Energy (Caltech-Crystalyx, UK) as described in Chapter 2 section 2.2.1. Delivery of the LMFL involved breaking the lick into small pieces to facilitate rapid consumption as previously conducted by Greenwood *et al.* (2000), Löest *et al.* (2001) and Leupp *et al.* (2005). Further details on supplement delivery are discussed in Chapter 2 section 2.4.1. At the beginning of each sampling period a representative sub-sample of all feed components were taken and stored at -20 ^{o}c for later chemical analysis of dry matter (DM), neutral detergent fibre (NDF), acid detergent fibre (ADF), ether extract (EE) and crude protein (CP) as described in Chapter 2 section 2.6 (Table 6.3). The NDF and ADF content of the block was not measured due to the trace quantity of fibre within the product.

Table 6.3: Chemical analysis of dietary components fed to sheep

	Ryegrass hay	Sugar beet	Low moisture feed lick
Dry matter (g/ kg)	912	897	996
Organic matter (g/ kg DM)	931	878	828
Crude protein (g/ kg DM)	72.6	97.4	129
Nitrogen (g/ kg DM)	11.6	15.6	18.8
Neutral detergent fibre (g/ kg DM)	694	414	ND
Acid detergent fibre (g/ kg DM)	379	181	ND
Ether extract (g/ kg DM)	18.3	3.40	95.3
Ash (g/ kg DM)	69.1	122	172

Low moisture feed lick - Crystalyx® extra high energy (Caltech-Crystalyx, Siloth, UK)
Sugar beet (Trident, AB Agri, Peterborough, UK)
ND = not determined

6.3.2 Appetite Study

The appetite study took place on days 10-14 of the experimental period (Table 6.2). It was felt that recording feed intake at the latter end of the adaptation period would allow for a better representation of voluntary forage intake. Each morning feed orts were collected from pens and individual daily forage intakes calculated by subtracting the initial feed offered from orts. Daily water intake was measured by recording the daily amount of water consumed from self-filling water bowls connected to a water meter. Daily live weight of the sheep was measured automatically via sheep entering the weigh crate of a Pig Performance Tester (Nedap, Groenlo, Netherlands) located in the

corner of each of their pens. Approximately 100g of sugar beet shreds (Trident, AB Agri, Peterborough, UK) were placed into the hopper of the feeder attached to the weigh crate and dispensed into feed troughs as an enticement for sheep to enter the crates.

6.3.3 Rumen Study

The rumen study was conducted on days 15 and 16 of the period (Table 6.2). The quantity of forage offered was restricted to 95% of each individual sheep's average forage intake recorded from days 9-14 to ensure full consumption of the ration. To prevent rapid consumption of the forage, forage was offered in two equal meals early morning and early afternoon.

On day 15 before morning feeding, four nylon bags filled with 8.8 g (8g DM) of Ryegrass hay were placed through the eye of the cannula and into the rumen for temporal incubation as described in Chapter 2 section 2.3. Two bags were removed after 4 hours and the remaining two bags after 24 hours of incubation and processed for drying as described in Chapter 2 section 2.3.2. The dry matter degradation of forage was calculated as described in Chapter 2 section 2.6.2. Only two time points were investigated due to the limited physiological capacity in the rumen for nylon bags and 4 and 24 hours were chosen to be in keeping with time points used in Chapter 4.

Rumen fluid samples were recovered on two consecutive days (15 and 16) at 0, 2, 4, 6, 8 and 24 hours post morning feeding as described in Chapter 2 section 2.2.2. Rumen fluid samples were processed and analysed for pH and molar concentrations of VFAs and ammonia as described in Chapter 2 section 2.5. Protozoa within rumen fluid samples were identified by light microscopy as described in Chapter 2 section 2.8.

6.3.4 Metabolism Study

The metabolism study took place on days 17–21 of the period (Table 6.2). Forage was restricted to 95% of each individual sheep's average forage intake as described in section 6.3.3.

Total tract digestibility of nutrients within diets including nitrogen balance was measured on days 18-21 of the period. Sheep were housed in individual metabolism crates fitted with a filter funnel system for the collection and separation of faeces and urine as described in Chapter 2 section 2.4.2. Each morning, feed orts water orts, total faecal and total urine voided were collected and processed as described in Chapter 2 section 2.4. A sub-sample of dietary ingredients and faecal material were taken and their nutritional composition analysed for DM, OM, NDF, ADF and N content as described in Chapter 2 section 2.6. The nutritional composition of the diet can be found in Table 6.3. A sub-sample of urine was recovered and the N content analysed as described in Chapter 2 section 2.6.6. Total tract

digestibility which included N balance was calculated as described in Chapter 2 section 2.4.2 and 2.4.3 respectively.

Enteric methane emissions from individual sheep were measured on days 19-21 of the period (Table 6.2) using individual indirect open circuit climate-controlled chambers as described in Chapter 2 section 2.4.4. Over the duration of the experiment climate within the chambers was approximately 14^{o}C and at 58% humidity. The flow rate of the chambers was on average 11.6 L/ second and the recirculation of air 182.7 L/ second allowing for approximately 2.1 air changes per hour. Calibration of the chambers demonstrated mean gas recovery to be 100 % ± 3 sem and the average concentration of methane in the atmosphere was 4.0 ppm.

6.4 Statistics

All statistical analysis was carried out in Genstat 19th edition. Data in the appetite study (section 6.3.2) and metabolism study (section 6.3.4) were analysed by analysis of variance (ANOVA) using the Latin Square design with sheep as rows and period as column. Data in the rumen study (section 6.3.3) were analysed by repeated measure ANOVA, with time as the repeated measure. The relative abundance of protozoa identified in rumen fluid was analysed via two-way ANOVA. Statistical significance was accepted at $p<0.05$.

6.5 Results

6.5.1 Appetite Study

The live weight of sheep was measured at the start and end of each period (Table 6.4). There was no significant difference between the live weights of sheep at the start or at the end of each period. Both diets had a positive effect on live weight suggesting animals to be in positive energy balance. Supplementing the diet with the LMFL tended to have an increasing effect on the live weight of sheep ($p=0.077$). Data were also analysed on a metabolic live weight ($LW^{0.75}$) basis, however there was no significant effect of LMFL supplementation on live weight. LMFL supplementation had no effect on voluntary forage or total dietary DM, OM, NDF or N intake. There was no effect of LMFL supplementation on the daily volume of water consumed.

Table 6.4: Effect of low moisture feed lick supplementation on appetite

	-LMFL	+LMFL	sed	p-Value
Live Weight (kg)				
Live weight start	86.2	84.7	1.074	0.255
Live weight end	86.9	87.3	0.976	0.715
Live weight gain/ loss	0.74	2.55	0.762	0.077
Forage intake (kg/ day)				
Dry matter	1.41	1.30	0.098	0.33
Organic matter	1.31	1.21	0.095	0.341
Neutral detergent fibre	0.98	0.89	0.080	0.363
Nitrogen	0.016	0.015	0.0006	0.158
Total dietary intake (kg/ day)				
Dry matter	1.50	1.46	0.098	0.718
Organic matter	1.39	1.35	0.095	0.660
Neutral detergent fibre	1.01	0.93	0.078	0.360
Nitrogen	0.017	0.018	0.0004	0.487
Water (L/ day)				
Intake	2.14	2.58	0.204	0.123

6.5.2 Rumen Study

The effect of LMFL supplementation on the DM degradation of forage *in-sacco* and rumen fermentation parameters were measured. LMFL supplementation had no effect on the DM degradation of forage *in-sacco* (Figure 6.2). Time had the effect of increasing the degradation of forage ($p<0.001$) in both treatments. There were no significant interactions between factors.

Supplementation of the diet with the LMFL had a tendency to reduce rumen pH ($p=0.055$), however the pH did not decrease below optimum pH for efficient rumen fermentation (Figure 6.3). Reasons for insignificance is likely to be due to large variations in the data reflected by large standard error of the mean. Time had a tendency to affect rumen pH which decreased between 0-8 hours and stabilised between 8 and 24 hours. There were no significant interactions between factors.

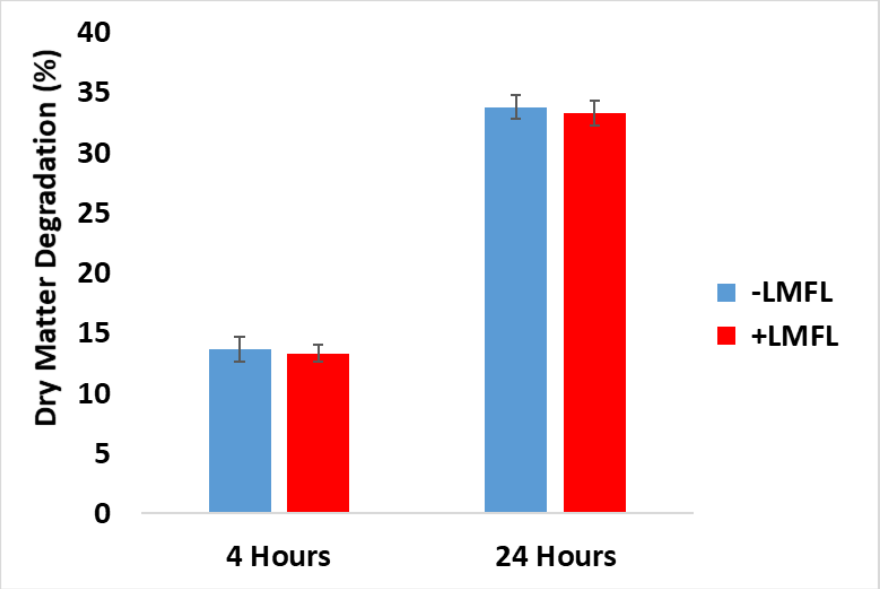


Figure 6.2: Effect of low moisture feed block (LMFL) supplementation on the dry matter degradation of forage *in-sacco*. Data are means of all sheep and error bars are standard error of mean. LMFL supplementation (p=0.969), Time (p<0.001), LMFL supplementation x Time (p=0.616).

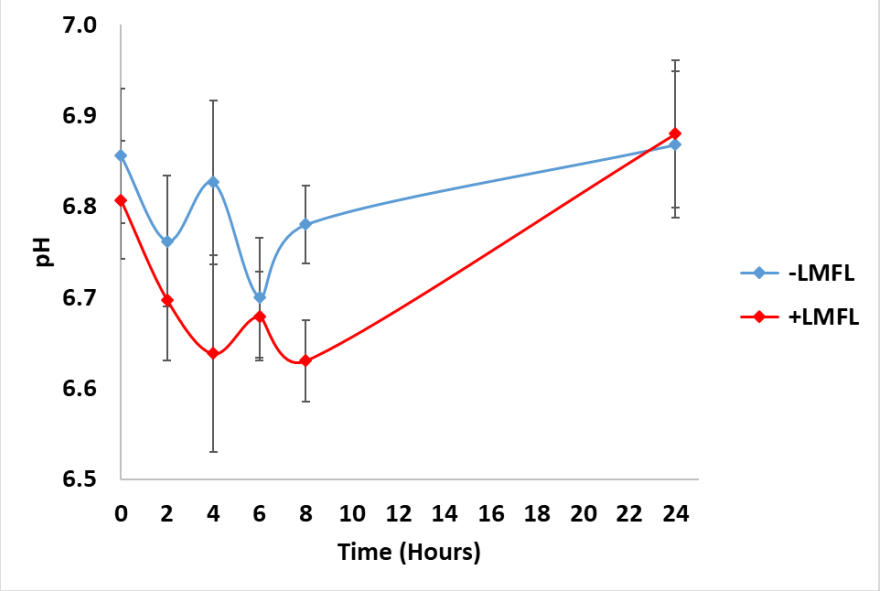


Figure 6.3: Effect of low moisture feed lick (LMFL) supplementation (-/+) on the temporal pH of the rumen post consumption of the LMFL. Data points (♦) are means of all six sheep and error bars are standard error of mean. LMFL supplementation (p=0.055), Time (p=0.066), LMFL supplementation x Time (p=0.410).

LMFL supplementation had no effect on the molar concentration of total VFAs detected within rumen samples (Figure 6.4). Likewise, time had no effect on the molar concentration of total VFAs and there was no significant interaction of the supplement and time. The individual molar concentration of VFAs were measured including the acetate to propionate ratio (A:P) as an indicator of the potential pathway of fermentation as described in Chapter 2 section 2.5.2 (Figure 6.5 A-E). LMFL supplementation had no effect on the molar concentrations of the major VFAs; acetate, propionate or butyrate or the branched chain VFAs (Figure 6.4 A-E). Likewise LMFL supplementation demonstrated no effect on A:P. Time had an effect on the molar concentration of propionate ($p<0.001$) which increased in concentration between 0-4 hours before decreasing and returning to a similar concentration to those observed before feeding at 24 hours. Time had an effect on the molar concentration of butyrate which increased in concentration from 0-8 hours before returning to a similar concentration to before feeding at 24 hours. Time had an effect on the molar concentration of the branched chain VFAs ($p<0.001$) which decreased in concentration from 0-8 hours before returning to a similar concentration as seen before feeding at 24 hours. Time had an effect on the ratio of A:P ($p<0.001$) which decreased between 0-8 hours before returning to similar concentration to that seen before feeding at 24 hours. There were no significant interactions between factors for any of the individual VFAs or A:P.

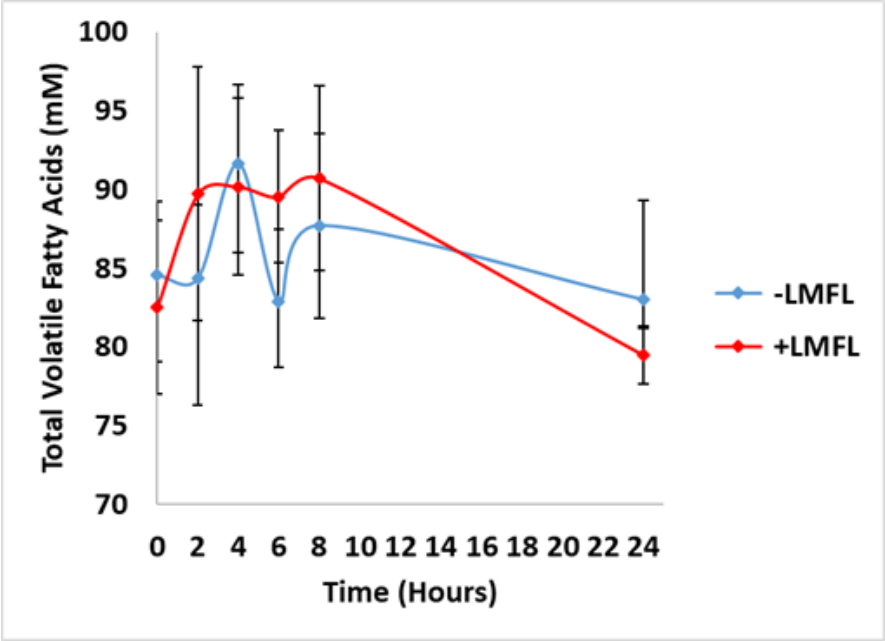


Figure 6.4: Effect of low moisture feed lick (LMFL) supplementation (-/+) on the total molar concentration (mM) of volatile fatty acids post consumption of the LMFL. Data points (♦) are means of all six sheep and error bars are standard error of mean. LMFL supplementation ($p=0.564$), Time ($p=0.233$), LMFL supplementation x Time ($p=0.505$).

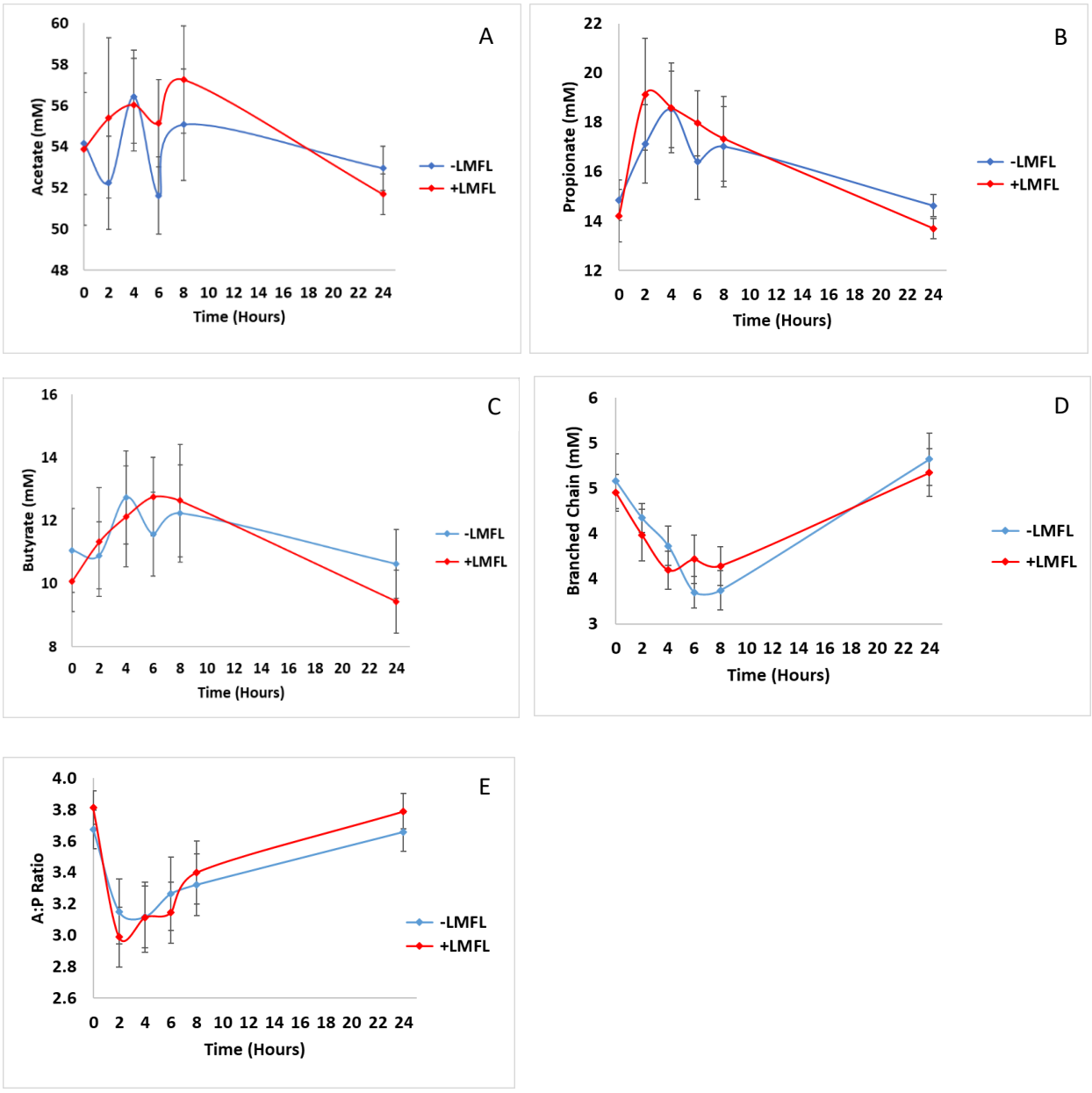


Figure 6.5: Effect of low moisture feed lick (LMFL) supplementation on the temporal molar concentration (mM) of individual volatile fatty acids, A) Acetate, B) Propionate, C) Butyrate, D) Branched Chain in the rumen and E) the ratio of acetate to propionate (A:P) post consumption of the LMFL. Data points (♦) are means of all six sheep and error bars are standard error of mean. Time Propionate, Butyrate, Branched Chain and A:P ($p<0.001$).

LMFL supplementation had no effect on the molar concentration of ammonia, although there was a tendency for the molar concentration to decrease when supplemented with the LMFL (Figure 6.6). Time had a decreasing effect on the molar concentration of ammonia from 0-8 hours before returning to pre-feeding concentration at 24 hours (p=0.003). There were no significant interactions between factors.

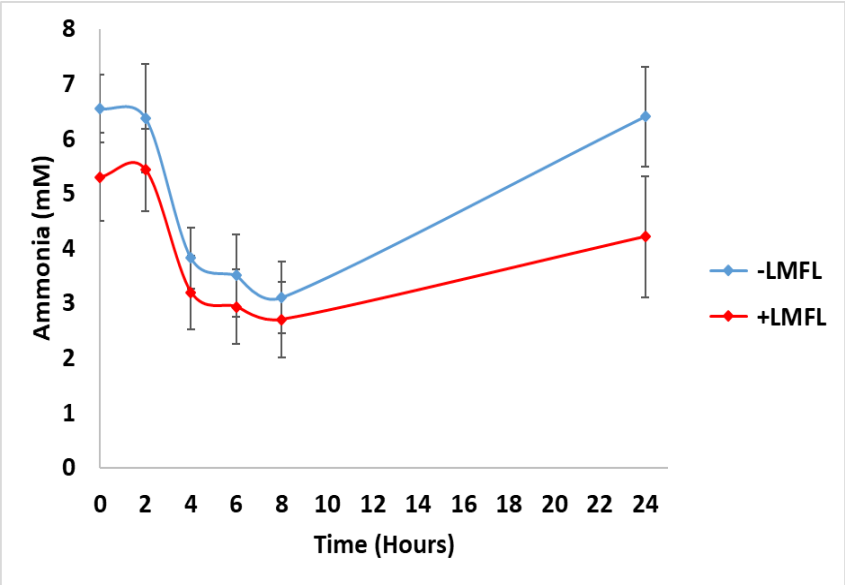


Figure 6.6: Effect of low moisture feed lick (LMFL) supplementation on temporal molar concentration of ammonia (mM) in the rumen post consumption of the LMFL. Data points (♦) are means of all six sheep and error bars are standard error of mean (n=6). LMFL supplementation (p=0.588), Time (p=0.003), LMFL supplementation x Time (p=0.461).

Protozoa present within rumen fluid were identified via their morphology under light microscopy and classified into the orders *Holotrichs*, *Entodiniomorphida* and "unidentified" where classification was not possible (Figure 6.7). There was no effect of LMFL supplementation on the total number of protozoa identified within 1 ml of rumen fluid or within the orders *Holotrichs* and *Entodiniomorphida.* Time had a significant effect on the total number of protozoa identified (p=0.006), with the number of protozoa classified into orders *Holotrichs* decreasing with time (p=0.011) and the number of *Entodiniomorphida* increasing with time (p=0.006). Moreover, the number of *Entodiniomorphida* appeared to increase at the expense of the *Holotrichs* at 24 hours. There were no interaction sbetween the supplement and time for the total number of protozoa identified or for protozoa classified into orders.

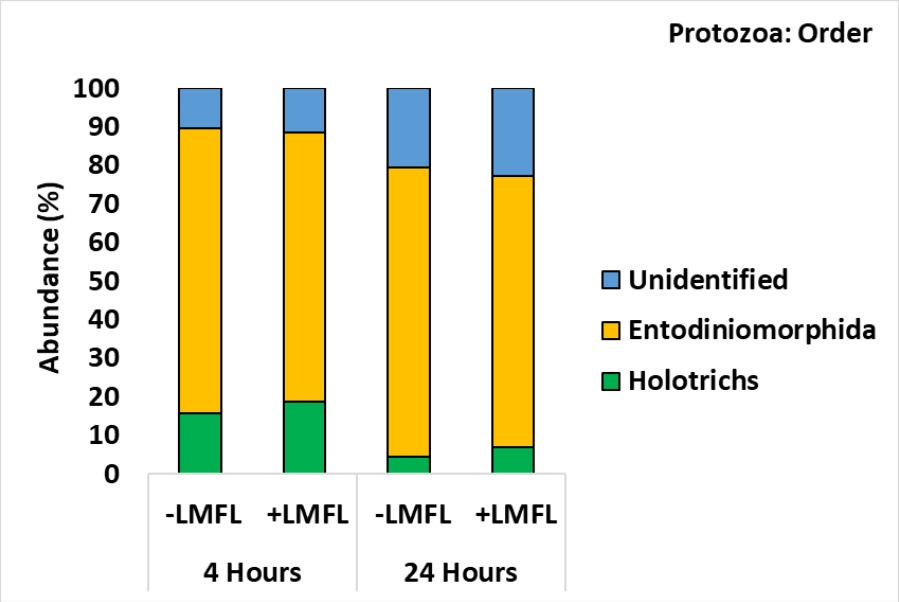


Figure 6.7: Effect of low moisture feed lick (LMFL) supplementation on the relative abundance of protozoa in 1 ml of rumen fluid classified into orders *Entodiniomorphida*, *Holotrichs* and unidentified. Data are means of all six sheep.

6.5.3 Metabolism Study

During the metabolism study total dietary intake, faecal output and urinary output were measured for the calculation of total tract digestibility of the diet (Table 6.5). LMFL supplementation had no effect on the total dietary DM, OM, NDF, ADF or N intake. Supplementation had no effect on the total faecal DM, OM, NDF or ADF voided. Likewise, there was no effect of LMFL supplementation on total faecal N or urinary N voided and sheep demonstrated to be in positive N balance. Thus, LMFL supplementation had no effect on the absorption of dietary DM, OM, NDF, ADF or N or the digestibility of dietary OM, NDF, ADF or N.

Table 6.5: Effect of low moisture feed lick supplementation on the digestibility of the diet

	-LMFL	+LMFL	sed	p-Value
Total dietary intake (kg/ day)				
Dry matter	1.22	1.24	0.051	0.718
Organic matter	1.13	1.14	0.050	0.868
Neutral detergent fibre	0.82	0.78	0.042	0.384
Acid detergent fibre	0.44	0.42	0.021	0.397
Nitrogen	0.014	0.026	0.0005	0.102
Total faecal and urinary output (kg/ day)				
Dry matter	0.46	0.44	0.012	0.254
Organic matter	0.41	0.39	0.011	0.185
Neutral detergent fibre	0.30	0.28	0.012	0.281
Acid detergent fibre	0.178	0.16	0.006	0.146
Nitrogen	0.012	0.013	0.0009	0.479
Absorption (kg/ day)				
Dry matter	0.76	0.79	0.042	0.437
Organic matter	0.72	0.74	0.042	0.558
Neutral detergent fibre	0.52	0.49	0.040	0.563
Acid detergent fibre	0.26	0.25	0.017	0.646
Nitrogen	0.002	0.003	0.001	0.790
Digestibility (g/ kg DM)				
Organic matter	582.4	599.1	12.10	0.240
Neutral detergent fibre	420.8	394.9	16.90	0.200
Acid detergent fibre	213.4	204.0	5.42	0.158
Nitrogen	1.54	2.09	0.949	0.594

Using individual indirect open circuit climate-controlled chambers, the daily enteric methane emissions of sheep were measured. LMFL supplementation had no effect on the daily enteric methane emissions or the daily enteric methane emissions per gram of dietary DM (Table 6.6).

Table 6.6: Effect of low moisture feed lick supplementation on daily enteric methane emissions

	-LMFL	+LMFL	sed	p-Value
Methane output (g/ day)	13.01	13.17	0.375	0.689
Methane output (g/ kg DM/ day)	11.66	10.85	0.358	0.086

6.6 Discussion

Previous research has demonstrated that cattle consuming low-quality forage-based diets supplemented with LMFLs to have the effect of increasing voluntary forage intakes and digestibility of the diet (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Leupp *et al.*, 2005, Chaudhry, 2008). The forages used in those studies were tropical forages typically grown in America such as; Prairie hay, Brome hay, Switchgrass hay and Alfalfa hay. To date there have been no studies investigating the effect of LMFL supplementation when feeding temperate forages typically used in livestock production systems in the UK or as fed to sheep. Therefore, in the studies reported here, sheep were fed a low-quality forage diet of Ryegrass hay (912 g DM; 931 g OM/ kg DM; 694 g NDF/ kg DM; 73 g CP/ kg DM). The chemical analysis of the forage revealed the hay to have a higher DM content and lower a lower CP content in comparison with typical reference temperate grass hays as described by (Ewing, 2016).

In this study sheep were fed the LMFLs as a flat rate dose each morning. A flat rate dose was provided rather than *ad-libitum* access to the LMFL to avoid over consumption as was discovered by Titgemeyer *et al.* (2004). Moreover, it was thought that if sheep had access to the LMFL on an *ad-libitum* basis it may result in the substitution of the forage with the LMFL due to is high palatability as a result of containing molasses. Likewise, a flat rate dose was fed to cattle in studies by Greenwood *et al.* (1998), Greenwood *et al.* (2000), Löest *et al.* (2001) and Leupp *et al.* (2005). Sheep rapidly consumed the LMFL supplement therefore the rumen study was conducted post consumption of the LMFL.

6.6.1 Appetite

The effect of LMFL supplementation on the appetite of sheep was measured. LMFL supplementation had no effect on voluntary DM, OM, NDF or N intake. Although there was no statistical significance between treatments there was a trend for sheep consuming the diet supplemented with the LMFL to have lower voluntary intakes in comparison with the control diet. A similar observation was made in

the metabolism study of this chapter. These results are in disagreement with Greenwood *et al.* (1998) who observed steers consuming a diet of Prairie hay *ad-libitum* supplemented with 450 g/ day of a LMFL to have increased voluntary OM, NDF and N intakes. Likewise, Greenwood *et al.* (2000) observed steers fed Prairie hay *ad-libitum* supplemented with 417 g/ day of LMFLs of base ingredients cane molasses, beet molasses or a concentrated separator by-product from the sugar industry to have an increasing effect on voluntary forage OM, NDF and N intakes. Löest *et al.* (2001) observed steers fed Prairie hay *ad-libitum* supplemented with 335 g/ day of LMFLs varying in non-protein nitrogen content (urea vs urea and biuret) to have a tendency to increase voluntary DM, OM, NDF and CP forage intakes. Likewise, Leupp *et al.* (2005) observed steers fed a diet of Switchgrass hay *ad-libitum* supplemented with 341 g/ day LMFLs with varying test additives (fermentation extracts, seaweed) to have a tendency to increase voluntary forage OM and CP intake.

Similar results to those observed in this chapter were made by Titgemeyer *et al.* (2004). Titgemeyer *et al.* (2004) observed steers fed forage-based diets *ad-libitum* of either; Alfalfa hay, Brome hay or a mix of Brome supplemented with 1.93 kg/ day of Alfalfa hay to have no effect on voluntary DM, OM, NDF or N intake when diets were supplemented with a LMFL. However, as indicated by the results presented here for sheep, intakes were lower for steers consuming the diet supplemented with the LMFL. In a separate study the effect of a low (CP, 144 g/ kg DM) and high protein (CP, 275 g/ kg DM) LMFL on the appetite of heifers was investigated. This study demonstrated heifers consuming forage-based diets *ad-libitum* of either; Prairie hay or Prairie hay supplemented with 1.96 kg/ day of Alfalfa hay to have no effect on the voluntary forage DM intake. Heifers consuming the high protein LMFL had greater total dietary DM intakes in comparison with the low protein and control diet, however this is most likely associated with heifers consuming a greater amount of the high protein LMFL in comparison with the low protein LMFL. Likewise, a trend was observed for diets supplemented with LMFLs to have a reducing effect on voluntary DM, OM, NDF and N intakes when heifers were supplemented with the low protein LMFL. Observations of reduced forage intake with supplementation may be associated with animals beginning to substitute forage with the LMFL rather than utilising it in addition to the forage or in complementation to forage as intended.

In this chapter, LMFL supplementation had a tendency to increase the live weight of sheep suggesting animals to be in positive energy balance. A similar observation was made by Titgemeyer *et al.* (2004) who observed heifers supplemented with a high protein LMFL to have greater average daily live weight gains in comparison with heifers supplemented with a low protein LMFL and the control diet. These observations may be indicative of the diet being sufficient for animal requirement and the addition of the LMFL aiding fat deposition. This suggests LMFL supplementation to only effect metabolism when the diet is nutritionally limiting. Therefore, it was a major limitation in this study to

include sugar beet in the diet, as sugar beet is a good quality feed high in protein and fermentable carbohydrates (Ewing, 2016). Moreover, due to there being a trend for LMFL supplementation to have an increasing effect on live weight but no effect on voluntary DM intakes, these observations may be a result of the additional energy the LMFL provides to the diet. The LMFL is retailed as an extra high energy supplement with a metabolisable energy content of 16 MJ/ kg DM and a sugar content of 380 g/ kg DM as disclosed by the manufacturer (Chapter 2 section 2.2.1). Therefore, increased live weight may have been associated with increased energy content of the diet provided by the LMFL. However, in this chapter the gross energy content of dietary components were not measured. Likewise, the gross energy of faecal and urinary excreta was not measured and it was not possible to measure the heat increment dissipated by animals in the climate controlled chambers. Therefore, further research is required to investigate the digestible and metabolisable energy content of the diet.

6.6.2 Forge Degradation and Total Tract Digestibility

The degradation of forage within the rumen was measured using the *in-sacco* technique, in which forage was incubated in the rumen of each sheep for 4 and 24 hours post consumption of the LMFL. Supplementing the diet with the LMFL had no effect on the dry matter degradation of forage. This in in agreement with the dual exchange *in-sacco* study and *in-vitro* study conducted in Chapter 5, which involved the *ad-libitum* supplementation of a diet of Ryegrass hay with the same LMFL used in this study. Likewise, an *in-sacco* study by Leupp *et al.* (2005) demonstrated LMFL supplementation to have no effect on the DM, NDF or ADF degradation of forage over 98 hours in the rumen of in cattle. However these observations disagree with *in-vitro* study by Chaudhry (2008) who demonstrated LMFL supplementation to increase the DM degradation of grass nuts and Barley straw incubated in buffered rumen fluid collected from slaughter cattle. Likewise, there was a trend for LMFL supplementation to increase the dry matter degradation of forage *in-vitro* in Chapter 4.

Total tract digestibility was measured by measuring total dietary intake, total faecal output and total urinary output and conducting chemical analysis of nutrients accordingly. LMFL supplementation had no effect on the total tract OM, NDF or ADF digestibility. Likewise, LMFL supplementation had no effect on N digestibility or N balance which was positive. This is in agreement with Titgemeyer *et al.* (2004) who observed heifers consuming a diet of Prairie hay *ad-libitum* supplemented with a low and high protein LMFL to have an increasing effect on digestibility, however there was no difference in the digestibility of the forage between supplements. When the nutritional composition of the diet was increased by supplementing Prairie hay with 1.96 kg/ day of Alfalfa hay the digestibility of the diet increased, however there was no effect of LMFL supplementation on digestibility. In a second study by Titgemeyer *et al.* (2004), LMFL supplementation had no effect on dietary DM, OM or NDF

digestibility of steers consuming diets of either Alfalfa hay *ad-libitum*, Brome hay *ad-libitum* or Brome hay *ad-libitum* supplemented with 1.94 g/ day Alfalfa hay. However, LMFL supplementation appeared to increase N digestibility, however this may be due to the additional protein provided to the diet in which the LMFL contained 332 g/ kg DM of CP of which contained no more than 12% of nitrogen from a non-protein nitrogen source. This agrees with Greenwood *et al.* (1998) who demonstrated steers consuming prairie hay ad-libitum supplementation with a LMFL to have increased N digestibility. Likewise, Greenwood *et al.* (2000) investigated the effect of LMFLs of varying base ingredients of either cane molasses, beet molasses or a concentrated separator by-product to have an increasing effect on the OM and N digestibility of steers consuming a diet of prairie hay *ad-libitum*. Supplementation with the beet molasses LMFL had an increasing effect on NDF digestibility however the other two LMFL did not. Löest *et al.* (2001) demonstrated steers consuming Prairie hay ad-libitum to have an increasing effect on DM, OM, NDF and CP digestibility when supplemented with LMFL of varying non-protein nitrogen contents. Leupp *et al.* (2005) demonstrated steers consuming Switchgrass hay ad-libitum to have an increased OM, CP, NDF and ADF digestibility when supplemented with LMFLs used as a carrier for various test additives. These observations, in conjunction with the observations made previously on appetite, are indicative of LMFL supplementation having no effect on metabolism when the diet is nutritionally sufficient. Moreover, the sheep used in this study were geriatric sheep that had long reached maturity and only had requirement for maintenance level, therefore it was likely a diet of Ryegrass hay and sugar beet was nutritionally sufficient. Therefore, future research is required to investigate the effect of LMFL supplementation on sheep that are actively growing or at physiologically demanding times.

Daily water consumption was measured via individual digital water meters attached to water troughs. Water consumption was in range of standard values for water consumption by sheep (2-3 L/ day) as described by (Czerkawski, 1986). LMFL supplementation had no effect on daily water consumption, although water consumption tended to be greater when diets were supplemented with the LMFLs. However, the water meters measured water consumption to the nearest litre and due to the sheep consuming low volumes of water measuring to the nearest ml would have given a much more precise measurement.

6.6.3 Rumen Fermentation

The effect of LMFL supplementation on rumen fermentation was investigated by measuring fermentation parameters, pH, the molar concentration of volatile fatty acids, ammonia and enteric methane emissions. Forage was consumed to appetite and in the rumen and metabolism study restricted to 95% of the previous days intake to ensure full consumption of the ration for the

metabolism trial. The forage was fed in two equal proportions am and pm therefore it was not possible to follow the pattern of fermentation post complete dietary consumption. This may be why the temporal pattern of fermentation parameters does not follow a particular pattern due to substrate being available at intervals throughout the day and therefore may mask the "true" effect of LMFL supplementation on temporal fermentation. However, animals consumed forage at a natural feeding pattern so is perhaps more representative in comparison with controlled measures such as using the *in-sacco* technique or restrictive feeding. Attempts were made to use temporal data as spot samples however no difference was observed between treatments.

LMFL supplementation had a tendency to reduce the pH of the rumen, however pH did not deviate below optimum pH for fermentation which is between 5 and 7.5 (Czerkawski, 1986). A drop in pH is most likely associated with the LMFL containing fermentable carbohydrates. However, there was no effect of LMFL supplementation on the total molar concentration of VFAs, or individual molar concentrations of the individual VFAs, acetate, propionate, butyrate or the branched chain VFAs. Likewise, Leupp *et al.* (2005) demonstrated steers consuming a forage-based diet of Switchgrass hay supplemented with a LMFL to have no effect on the pH of the rumen or the molar concentrations of total VFAs, acetate, propionate or butyrate. These results disagree with observations made *in-vitro* in Chapters 4 and 5, in which LMFL supplementation resulted in increased molar concentrations of total VFAs; acetate, butyrate and propionate. However, pre-adapting the rumen to LMFL supplementation in Chapter 5 had no effect on the molar concentrations of VFAs. Greenwood *et al.* (1998) observed steers consuming a forage-based diet of Prairie hay supplemented with LMFLs of varying base ingredients to have different effects on rumen fermentation. Supplementation of the diet with a cane molasses LMFL decreased rumen pH but had no effect on the molar concentration of total VFAs or the proportions of individual VFAs. However, supplementation with a LMFL with base ingredient of beet molasses decreased rumen pH and had an increasing effect on the molar concentration of total and proportions of individual VFAs, acetate, propionate, butyrate. Likewise, supplementation of the diet with a concentrated separator by-product based LMFL had no effect on rumen pH but increased the molar concentration of total VFAs, acetate and propionate. This suggests the specification and dietary ingredients within LMFLs to affect fermentation, thus making comparisons between studies difficult.

LMFLs add additional protein to the diet from dietary ingredients such as molasses, sugar beet and urea. The LMFL used in this study had a crude protein content of 120 g/ kg DM of which contained 5% protein equivalent of urea (Chapter 2 section 2.2.1). Inclusion of urea in the diet has been demonstrated to increase rumen ammonia concentration (Cameron *et al.*, 1991). Urea is rapidly hydrolysed by rumen microbes for deamination into ammonia (Kingston-Smith *et al.*, 2008) and provided the fermentable metabolisable energy content of the diet is sufficient can be utilised by

microbiota to produce microbial cell protein. It was hypothesised that LMFL supplementation would have an increasing effect on the molar concentration of ammonia soon after consumption as a result of the additional rumen degradable protein provided to the diet. Greenwood *et al.* (1998) observed a peak in ruminal ammonia concentration 2 hours post feeding of a LMFL to steers. However, in this chapter LMFL supplementation had no effect on the molar concentration of ammonia. This observation agrees with those made *in-vitro* in Chapters 4 and 5. However disagrees with observations made by Leupp *et al.* (2005) who demonstrated LMFL supplementation to have an increasing effect on ruminal ammonia concentration. However, the crude protein content of the LMFLs (CP, 400 g/ kg DM) was greater in comparison with this study. Greenwood *et al.* (1998) observed LMFL supplementation to increase the molar concentration of ammonia. Likewise, the crude protein content of the LMFLs fed (CP, 300 g/ kg DM) were greater in comparison with this study and had a higher content of non-protein nitrogen (12%). These results suggests the formulation and dietary ingredients within LMFL to play a role in affecting the metabolism of sheep and LMFL to be a good carrier for adding nutrition to the diet. Preparations at sample collection (inclusion of sulphuric acid in urine collection trays) were made to measure purine derivatives as described by Chen & Gomes (1992) for estimation of microbial cell protein. However, it was decided not to analyse samples via HPLC due to the LMFL supplementation having no effect on any other previous measures associated with protein metabolism in this trial.

Methane is a by-product from the fermentation of plant material by microbiota in the rumen (McDonald *et al.*, 2010). Approximately 80% of all methane emitted by ruminants is produced within the rumen with the remainder 20% generated from faecal material (Vergé *et al.*, 2007). Studies investigating the effect of the diet on methane emissions are of great interest regarding methane being a potent greenhouse gas. Daily methane emissions in this chapter were measured using indirect open circuit climate-controlled chambers (No Pollution Ltd, Edinburgh, UK). LMFL supplementation demonstrated no effect on the total daily methane emission or the total daily methane emission expressed per kg of dry matter. This is perhaps unsurprising as the LMFL demonstrated no effect on the voluntary forage intake, dry matter degradation of forage in the rumen, total tract digestibility or rumen fermentation. Using the SF6 technique, Hart & Newbold (2015) observed heifers extensively grazing in the autumn and spring supplemented with LMFLs *ad-libitum* to have no effect on daily methane emissions. However, heifers supplemented with the LMFL had increased live weight gain and when methane was expressed per kg of live weight gain, LMFL supplementation was effective in decreasing methane emissions.

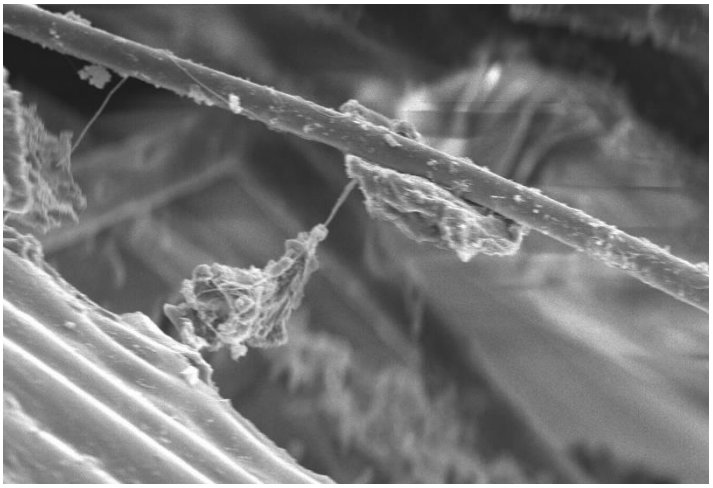
Protozoa within rumen fluid samples were identified via light microscopy. It was decided to categorise protozoa into the orders; *Holotrichs* or *Entodiniomorphida* and not refine identification taxonomically

further due to difficulties in identification via light microscopy. Abe & Iriki (1989) observed the population of *Holotrichs* to increase in response to the provision of reducing sugars provided to the diet. Therefore, a similar response was expected in this study due to the LMFL having a high sugar content of 380 g/ kg DM in which the majority of the sugar is sucrose. LMFL supplementation had no effect on the total protozoal count or the total count of protozoa in the orders *Holotrichs* or *Entodiniomorphida*. Time had the greatest effect on protozoal count where the number of *Holotrichs* decreased in number at the expense of *Entodiniomorphida which* increased with time. Williams (1986) discusses there to be a diurnal pattern in the abundance of protozoal population with the abundance of *Holotrichs* decreasing 12-20 hours post feeding and the *Entodiniomorphida* population decreasing for up to 16 hours after feeding before returning to initial pre-feeding level. Abe *et al.* (1981) attributes the decrease in the abundance of the *Holotrich* population present in rumen fluid due to protozoa adhering to the epithelium of the reticulum post feeding and then returning to the rumen pre-feeding. However, forage in this study was consumed by sheep to appetite, therefore this observation may be resultant of the LMFL thus further research is required to determine this.

6.7 Conclusion

In conclusion, supplementing the diet of mature non-productive sheep with a high energy LMFL had no effect on voluntary forage intake, digestibility of the diet or rumen fermentation. However, there was a trend for LMFL supplementation to result in increased live weight gain of sheep. This suggests that LMFL supplementation has no effect on metabolism when the diet is nutritionally sufficient for maintenance. Further research is required to determine the effect of LMFL supplementation on the metabolism of productive sheep with a greater nutritional demand.

Chapter 7 General Discussion and Overall Conclusion



7.1 Introduction

Low moisture feed licks (LMFL) are a popular commercial feed supplement for ruminants in extensive grazing systems. Feed licks are placed out at pasture for self-regulatory consumption and thus require less labour expenditure in comparison to traditional forms of supplementation such as concentrate feeds. Feed licks are retailed as forage balancers and provide additional energy, protein, vitamins and minerals to the diet, which otherwise may be lacking in forage. Moreover, the hardness of the LMFL (> 95% DM), attributed to its manufacturing process, prevents overconsumption and spoilage from the elements.

Previous research has demonstrated that cattle consuming poor-quality forages supplemented with LMFLs to have increased voluntary forage intakes and to increase the digestibility of the diet (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Leupp *et al.*, 2005, Chaudhry, 2008). Moreover, LMFL supplementation has demonstrated positive effects on animal performance in comparison with control animals, in terms of animals having greater live weight gains (Hart & Newbold, 2015), improved body condition scores and blood metabolic status (Cabiddu *et al.*, 2014). The biological mechanism by which LMFLs effect animal performance is not fully understood. In this thesis it was hypothesised LMFLs would have a positive effect within the rumen, whereby rumen microbiota are stimulated thus increasing the degradation and fermentation potential of forage and in turn resulting in greater voluntary forage intakes and improvements on animal performance. Through a series of *in-vitro*, *in-sacco* and *in-vivo* experiments the overall aim of this thesis was to investigate the effect of LMFL supplementation on metabolism to better explain the outcomes observed in animal performance. Previous research associated with LMFLs have largely been focused on cattle consuming poor quality forages. Therefore, this thesis aimed to investigate the effect of LMFL supplementation in sheep to see if the effects of LMFLs could be considered comparable. The LMFL used in studies throughout this thesis was Crystalyx® Extra High Energy (Caltech-Crystalyx® UK, Siloth, UK).

7.2 LMFL Supplementation for Improving Animal Performance

Lamb production in the UK is seasonal, largely a result of ewes being short day breeders. The lambing season typically spans from February – April with lambs going off for slaughter when they are commercially viable, generally at 5 – 8 months of age (NSA, 2020). The price of lamb (£/ kg) is volatile and fluctuates according to supply and demand, therefore it is essential that production systems are efficient for lambs to achieve slaughter weights at a suitable time for satisfactory return.

Nutrition is an integral part of any livestock production system with nutrition accounting for a large proportion of on farm variable costs. Over-nutrition can be economically damaging as well as impeding

the health of stock with negative effects on animal performance such as poor reproductive performance, increased incidence of uterine prolapse and the onset of metabolic diseases such as ketosis. Likewise, under-nutrition can result in setbacks in animal performance, where undernourished animals can display poor reproductive performance in terms of low scanning percentage and increased days to conception post ram turn out, thereby extending the lambing period. Poor nutrition throughout gestation can result in low lamb birth weights and complications post-partum. For example, the onset of twin lamb disease, poor colostrum quality, poor milk yield and quality can affect lamb survival, viability and prolonging of the time taken to reach commercial viability for slaughter. Likewise, undernourished ewes can display poor maternal ability in which they have preference of feeding over rearing their lambs and can prematurely dry up. This increases the likelihood of lambs being reared artificially which is of great cost to the producer in terms of feed (milk replacer, creep feed) and labour requirement. Therefore, it is important that nutrition and nutritional management is sufficient to cater for all animal maintenance and production requirements whilst being economically viable. Recording the performance (live weight, body condition, scanning data) of sheep within a flock is important in understanding how the flock is performing on a whole, identifying animals that are underweight for target feeding and highlighting any key areas in the production system for which improvements can be made to increase overall production efficiency.

Previous research has demonstrated supplementation of forage-based diets with LMFLs to have positive effects on animal performance (Cabiddu *et al.*, 2014, Hart & Newbold, 2015). However, research investigating the effect of LMFL supplementation on animal performance in a commercial environment and in sheep is limited. Therefore, the effect of LMFLs on the performance of a commercial flock of breeding ewes extensively grazing in the uplands was investigated (Chapter 3) (Figure 7.1). This small-scale study demonstrated LMFLs to have positive effects on the performance of breeding ewes in terms of increased live weight gains, which is a similar observation to that previously made in cattle (Hart & Newbold, 2015). Moreover, LMFL supplementation demonstrated positive effects on the reproductive performance of ewes who had a tendency to have a greater scanning percentage as a result of an increased number of twin bearing ewes and a shorter lambing period in comparison to the control group, suggesting conception within the first oestrous at tupping. Moreover, there was a trend for lambs born to dams supplemented with the LMFLs to reach commercial viability for slaughter quicker in comparison to the control group. The results of this study demonstrate LMFLs to be an effective supplement for improving the performance of stock in a commercial environment. However, it must be remembered that this study was a small-scale study with a limited number of animals thereby making statistical analysis limited. In addition, in this study and throughout this thesis the effect of the trace elements alone on animal performance were not

investigated. Therefore, this study would need repeating on a larger scale with increased control measures put in place before making any firm conclusions.

The performance of mature sheep as a result of LMFL supplementation was also measured (Figure 7.1). The live weight of sheep at the start and end of experimental periods was recorded and live weight change calculated (Chapters 5 and 6). In contrast to the commercial trial, the sheep in these studies were non-productive animals (date of birth; 2012) that only had a nutritional requirement for maintenance level. No weight loss was observed in any of the experiments for sheep consuming the control or treatment diets suggesting sheep to be in positive energy balance and the diet alone sufficient for maintenance requirement. When the LMFLs were available to sheep *ad-libitum* in a group environment, supplementation had no effect on the live weight (Chapter 5). However, when the LMFL was fed as a flat rate dose to sheep housed in individual stalls there was a tendency for sheep to gain weight (Chapter 6). The LMFL used in this study was a high energy supplement with a total sugar content of 380 g/ kg DM and a metabolisable energy content of 16 MJ/ kg DM therefore it is not surprising weight gain occurred (section 7.3.2). These observations suggest that when the diet alone is nutritionally sufficient for animal requirement LMFL supplementation to perhaps be a useful supplement to increase the live weight of sheep for example for flushing of ewes and rams before tupping or in preparation for live weight loss over the winter months.

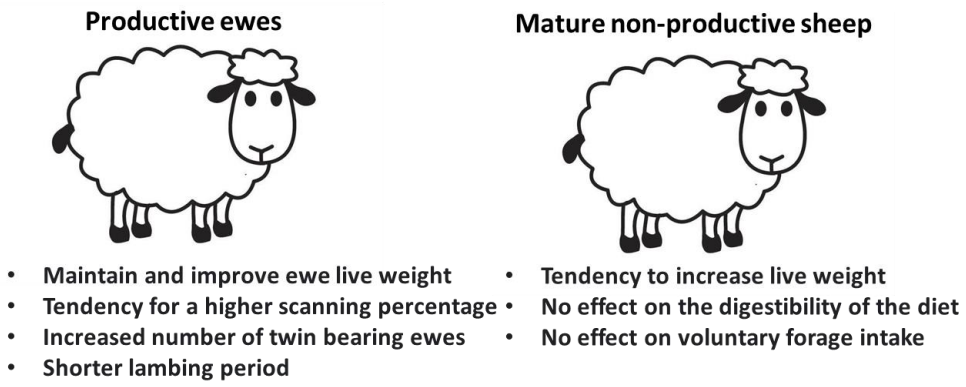


Figure 7.1: Schematic diagram demonstrating the key findings for the effect of low moisture feed lick supplementation on the performance of mature non-productive sheep housed indoors fed Ryegrass hay ad-libitum and productive ewes extensively grazing in the uplands.

7.3 Effect of LMFL Supplementation on Rumen Function

Ruminants have a unique digestive system in which the stomach consists of four chambers, the largest being the rumen (McDonald *et al.*, 2010). The rumen is host to a diverse range of microbiota capable of producing and secreting enzymes allowing for the degradation of plant structural carbohydrates that are otherwise indigestible by the mammalian stomach (Huws *et al.*, 2018). It was hypothesised that LMFL supplementation would have a positive effect on the rumen, whereby microbial metabolism would be enhanced as a result of increased enzyme production, which in turn would increase the degradation and fermentation potential of forage. Therefore, the effect of LMFL supplementation on rumen function was investigated using *in-vitro* and *in-sacco* techniques.

Traditionally experiments investigating the foregut degradation and fermentation of feedstuffs have been carried out *in-sacco*, however as Mohamed & Chaudhry (2008) discuss, *in-vitro* techniques offer an alternative method that is less costly and time consuming in comparison. The *in-sacco* technique involves the incubation of forage within the rumen for allotted time intervals and takes into account the animal effect such as; rumen flow, saliva production and the absorption of fermentation products. Whilst the *in-vitro* technique involves the incubation of forage in buffered rumen fluid in a closed system (Theodorou *et al.*, 1994). However, it must be noted that forage delivery in both techniques is not how forage would naturally enter the rumen. These techniques do not include damage to plant cells that would occur via mastication, which is an important part of the digestive system which causes damage to plant cell structures, reduces the tensile strength of tissues and increases the surface area of plant material thus improving microbial access to dietary substrate (Varga & Kolver, 1997, Kingston-Smith & Thomas, 2003).

7.3.1 Forage Degradation in the Rumen

The effect of LMFL supplementation on the dry matter degradation of Ryegrass hay within the rumen was measured *in-vitro* using the gas production technique and *in-sacco* in mature non-productive sheep. It was hypothesised that when the LMFL was fed as a flat rate dose the supplement would have the effect of increasing the degradation of forage within the rumen as a result of the LMFL providing a substrate to stimulate the metabolic activities of rumen microbiota. Moreover, it was hypothesised that pre-conditioning the rumen to the LMFL would increase forage degradation as a result of the microbial population having a greater metabolic activity because of the additional nutrition the LMFL provides to the diet in the long term.

LMFL supplementation had no effect on the percentage of forage degraded or the rate of forage degradation over 48 hours *in-vitro* or *in-sacco*, when formulated into the diet based on the estimated voluntary intake of the LMFL by sheep in the study (175 g/kg DM) (Chapter 5) or as a flat rate dose (70

g/ day) (Chapter 6). This is a similar observation to that made *in-sacco* in cattle by Leupp *et al.* (2005). Likewise, pre-conditioning of the rumen to the LMFL had no effect on the dry matter degradation of forage or rate of dry matter degradation *in-vitro* or *in-sacco* (Chapter 5). This suggests the LMFL to have neither a short nor long term effect on the degradation of forage within the rumen of mature non-productive sheep. However, there was a trend for LMFL supplementation to increase the dry matter degradation of forage over 24 hours when the supplement was formulated into the diet based on the manufacture's recommendation (36 g/ kg DM) (Chapter 4), which is a similar observation made *in-vitro* by Chaudhry (2008) using rumen fluid collected from slaughter cattle. These results are indicative of the LMFL having no effect on the dry matter degradation of forage in the rumen of mature non-productive sheep with only a nutritional requirement for maintenance. Therefore, further research is required to determine the effect of LMFL supplementation on the degradation of forage within the rumen of growing animals or mature animals with a nutritional requirement for maintenance plus production (breeding).

7.3.2 Voluntary Forage Intake and Total Tract Digestibility

Studies investigating the effect of LMFL supplementation on voluntary feed intakes and the total tract digestibility of tropical forages have been conducted in growing cattle *in-vivo*. Studies have demonstrated LMFL supplementation to have the effect of increasing voluntary forage intakes and the total tract digestibility of the diet (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Leupp *et al.*, 2005) and thus result in improvements in animal performance as a result of increased utilisation of nutrients from forage. It was therefore hypothesised that LMFL supplementation would exhibit similar effects in sheep *in-vivo* as a result of the LMFL stimulating rumen microbiota and thus increasing the degradation and fermentation potential of forage.

The effect of LMFL supplementation fed as a flat rate dose (70 g/ day) was investigated on the metabolism of mature non-productive sheep, consuming a forage-based diet typical of use in agriculture in the UK (Chapter 6). LMFL supplementation had no effect on the voluntary forage or total dietary, DM, OM, NDF or N intake, which is in agreement with studies investigating the effect of LMFLs on the degradation of forage within the rumen as discussed in section 7.3.1. Likewise, LMFL supplementation had no effect on the OM, NDF or N digestibility of the diet. However, there was a trend for sheep consuming the diet supplemented with the LMFL to have slightly lower voluntary forage intakes in comparison with the control group, which is a similar observation made by Titgemeyer *et al.* (2004) in steers consuming tropical forages. Likewise, in the study by Titgemeyer *et al.* (2004), LMFL supplementation had no effect on the DM, OM or NDF digestibility of the diet. These results may be indicative of animals beginning to substitute the forage component of the diet for the

LMFL rather than utilise it in addition or in complementation to forage as intended. This is perhaps unsurprising regarding the forage being poor quality and the LMFL having a high palatability due to containing molasses and having a high sugar (380 g/ kg DM) and energy content (16 MJ/ kg DM).

As discussed in section 7.2 sheep did not lose any live weight when fed the control or treatment diets, however there was a tendency for sheep supplemented with the LMFLs to gain weight. This is perhaps indicative of the diet being sufficient for maintenance requirement and for LMFL supplementation to only effect metabolism when the diet is nutritionally limiting. A similar conclusion was made in heifers by Titgemeyer *et al.* (2004), whom investigated the effect of a low (CP, 144 g / kg DM) and high (CP, 275 g/ kg DM) protein LMFL on the metabolism of heifers consuming tropical forage-based diets varying in nutritional quality. Here, LMFL supplementation had the effect of increasing the digestibility of the poorer quality forage-based diet but had no effect on the digestibility of the better-quality forage-based diet. In addition, heifers consumed a greater amount of the LMFL when fed the poorer quality diet. Heifers also had greater intakes of the high protein LMFL in comparison with the low protein LMFL suggesting affinity for protein in the diet, which perhaps explains why total dietary and forage dry matter intakes and the live weight gain of heifers was greater in comparison with the control and low protein LMFL diet.

Little research has been conducted on the utilisation of LMFLs by ruminants extensively grazing. However, from the findings discussed above it could be hypothesised that consumption of the LMFLs would be greatest when grazing is limiting in nutritional quality and availability. The utilisation of LMFLs by commercial breeding ewes grazing in the upland was investigated (Chapter 3). This study demonstrated a trend for consumption of the LMFLs by ewes to be greatest when sward height was low in the winter and during the last trimester of gestation when the nutritional requirement was high. This suggests that animals were regulating the consumption of LMFLs in accordance to nutritional requirement. Likewise, sheep tend to consume more of the LMFL in the winter when grazing is limited in comparison with the spring when grass growth reinstates, however this observation is based on anecdotal evidence and further research is required to fully understand the seasonal utilisation of LMFLs in relation to nutritional requirement.

Collectively, these results are indicative of LMFL supplementation only affecting animal appetite and the digestibility of the diet when the diet is nutritionally limiting. However, LMFL supplementation has the potential to increase the live weight gain of ruminants regardless of having no effect on rumen metabolism. This is most likely attributed to the high energy and sugar content of the LMFL aiding fat deposition. It is well known that the consumption of sugar results in increased blood glucose concentration and in turn insulin secretion from the pancreas. Insulin has the effect of increasing

glucose uptake by muscle and fat cells, promoting protein synthesis and lipogenesis (Garnsworthy *et al.*, 2008). Schoonmaker *et al.* (2003) suggests that the feeding of a high starch diet to increase the production of propionate and thus insulin secretion may result in increased glucose uptake by peripheral cells and in turn fat deposition with the potential of improving carcass marbling scores. Therefore, the effect of LMFL supplementation having a tendency to increase live weight gain of mature sheep (Chapter 6) may be a result of a spike in insulin concertation induced on consumption of the LMFL thus promoting lipogenesis. Moreover, manipulation of the diet to elevate plasma insulin secretion has demonstrated positive effects on the reproductive performance of dairy cows. Gong *et al.* (2002) demonstrated high producing dairy cows fed diets to elevate plasma inulin concentrations early in lactation to improve follicular development post-partum. Similar benefice was observed by Garnsworthy *et al.* (2009) who demonstrated dairy cows fed diets to increase plasma insulin concentrations early in lactation to improve the onset of the first oestrous post-partum. Moreover, in this thesis (Chapter 3) LMFL supplementation had the effect of improving the reproductive performance of ewes grazing in the uplands (section 7.2). Therefore, further research is required to determine the endocrinological effects of LMFL supplementation on animal metabolism and reproductive performance.

7.3.3 Rumen Fermentation

Microbiota resident in the rumen have the capability of fermenting dietary components into products of utility to both the host ruminant and microbiota (Kumar *et al.*, 2015). The effect of LMFL supplementation on rumen fermentation was measured *in-vitro* using the gas production technique. It was hypothesised that LMFL supplementation would have an enhancing effect on fermentation within the rumen as a result of increased microbial activity. Likewise, it was hypothesised that pre-adapting the rumen to the LMFL would have an enhancing effect on fermentation as a result of microbiota being stimulated when a continuous supply of additional nutritional to the diet was provided.

Notably, in the absence of dietary substrate, LMFL supplementation had the effect of increasing the total cumulative volume of gas produced (Chapter 4). A similar observation was made in the presence of substrate but to a greater extent (Chapters 4 and 5). Moreover, increasing dosage of the LMFL had an increasing effect on the volume of gas produced both in the absence and presence of substrate. However, pre-adapting the rumen to the LMFL had no effect on gas production (Chapter 5). This is indicative of the LMFL being utilised as a substrate directly by rumen microbiota for fermentation. This is perhaps unsurprising regarding the high sugar content (380 g/ kg DM) of the LMFL and the base

ingredient of the LMFL to be molasses which has a high fermentable carbohydrate content (Ewing, 2016).

It was not possible to measure the percentage of individual gases produced (carbon dioxide, methane) in the experiments reported here. However, the total daily methane emissions of sheep was measured *in-vivo* using individual indirect open circuit climate-controlled chamber (No Pollution Ltd, Edinburgh, UK) (Chapter 6). It was hypothesised that the LMFL would increase the digestibility and voluntary intake of forage and thus result in increased methane emissions. However, the study demonstrated no effect of LMFL supplementation on the daily enteric methane emissions or daily enteric methane emissions per kg of dietary DM. Likewise, in this study LMFL supplementation had no effect on voluntary forage intake, digestibility of the diet or on rumen fermentation. This observation is in agreement with Hart & Newbold (2015) who using the SF_6 technique demonstrated LMFL supplementation of heifers grazing in the autumn and spring to have no effect on daily enteric methane emissions. This study also demonstrated heifers to have increased live weight gains in comparison with control animals however it was not known what the voluntary forage intakes were or the effect of the supplement on the digestibility of the diet. As discussed in section 7.3.2 LMFL supplementation appears to effect metabolism when the diet is nutritionally limiting, therefore further research should be conducted to investigate the effect of LMFL supplementation on enteric methane emissions when the diet is nutritionally limiting.

Fermentation parameters and products (pH, VFAs and ammonia) were measured in rumen inoculum as an indication of fermentation. As mentioned previously, a buffer was used in *in-vitro* incubations (Chapter 4 and 5) to maintain pH and therefore change in pH did not occur as a result. However, pH was measured *in-vivo* (Chapter 6) and demonstrated LMFL supplementation to have a tendency to lower rumen pH, although pH did not drop below the optimum pH for rumen fermentation. This outcome is probably due to the LMFL having a high fermentable carbohydrate content in which the total sugar content was 380 g/ kg DM. However, reasons why pH did not decrease below optimum pH for fermentation may be associated with the supplement being fed as a flat rate dose. Likewise, the consumption of the lick is thought to induce saliva production due to its dehydrated nature however this is anecdotal evidence. The effect of LMFL supplementation on rumen pH has previously been conducted in cattle *in-vivo*, however there are inconsistencies between studies. Likewise, there are inconsistencies between studies for the effect of LMFL supplementation on the production of VFAs. This is most likely associated to differences in inclusion rate of the LMFL within the diet, different specifications of the LMFLs and different inclusion rates of dietary ingredients within the LMFLs.

Volatile fatty acids are a product of microbial fermentation of dietary substrate and the driver of pH change. In the absence of dietary substrate LMFL supplementation increased the molar concentration of total VFAs *in-vitro* (Chapter 4). Similar observation was made in the presence of substrate but to a greater extent (Chapters 4 and 5). However, pre-adaptation of the rumen to the LMFL had no effect on the total molar concentration of VFAs (Chapter 5), which is in correspondence with the gas production data discussed above. Moreover, these observations confirm the use of the LMFL as a substrate for microbial fermentation and for the LMFL to have no long-term effect on microbial metabolism.

The individual molar proportions of VFAs were investigated as an indication of the potential pathways of fermentation. LMFL supplementation in both the presence and absence of substrate had the effect of increasing the major VFAs, acetate, propionate and butyrate *in-vitro* (Chapter 4, 5) suggesting the LMFL to increase fermentation on the whole. However, pre-adapting the rumen to LMFL supplementation had no effect on the molar concentration of the major VFAs. This further confirms the effect of the LMFL enhancing rumen fermentation but having no lasting effect. However, such observations were not made *in-vivo* when sheep were fed a flat rate dose of the LMFL (Chapter 6). This is most likely a result of the LMFL having a short-term effect within the rumen and for the supplement to be rapidly utilised by microbiota for fermentation.

There was no effect of LMFL supplementation on the molar concentration of ammonia *in-vitro* or *in-vivo* (Chapters 4, 5 and 6). This disagrees with Greenwood *et al.* (2000) and Leupp *et al.* (2005) who both observed LMFL supplementation to increase the molar concentration of ammonia. However, the protein content of the LMFLs used in these studies were a lot higher in comparison with the LMFL used in this study and had higher non-protein nitrogen contents.

Overall, these results suggest LMFLs to have an additive effect within the rumen whereby it acts as a substrate for fermentation by rumen microbiota (Figure 7.2). This is of benefit to the host in that the products of fermentation (VFAs) are of utility to the host and perhaps explains why improvements on animal performance are observed. However, pre-adapting the rumen to the LMFL had no effect on fermentation suggesting the supplement to be fermented at the point of consumption and there to be no lasting effects on microbial activity highlighting it's use as a long-term supplement rather than as a short-term supplement. Moreover, differences in the specification and ingredients of LMFLs are likely to influence the effect and the extent the supplement has on fermentation therefore residing in variation between products. It must be remembered that LMFLs are a form of supplement with each product having a different nutritional specification and dietary ingredients, therefore comparisons between studies can be somewhat limited. This was one of the main reasons the same LMFL was used

throughout this thesis. Therefore, further research is required to exactly pinpoint which aspect of the LMFL is responsible for affecting metabolism when the diet is nutritionally limiting for example, sugar, protein, vitamins or minerals.

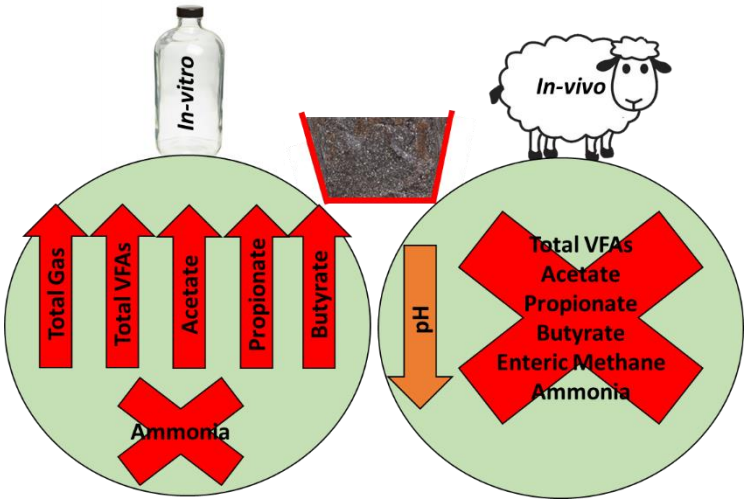


Figure 7.2: Schematic diagram demonstrating the effect of LMFL supplementation on rumen fermentation in mature non-productive sheep *in-vitro* and *in-vivo*. Arrows indicate the direction of fermentation and x represents no effect. The colour orange represents a tendency for an effect.

7.4 Rumen Microbiota

The rumen microbiome is host to a diverse range of microbiota across several kingdoms such as, bacteria, archaea, anaerobic fungi, protozoa and bacteriophages (Huws *et al.*, 2018). These microorganisms work in symbiosis with the host ruminant and in synchrony with one another to digest and ferment plant material that is otherwise indigestible by mammals (Kumar *et al.*, 2015). To date there have been no studies investigating the effect of LMFL supplementation on rumen microbiota.

It was hypothesised that LMFL supplementation would have a stimulating effect on rumen microbiota and pre-adapting the rumen to the LMFL would result in a more "active" population capable of increasing the degradation and fermentation potential of forage. To test this hypothesis the effect of LMFL supplementation on microbial biomass was measured using the quantitative polymerase chain reaction technique (q-PCR) (Chapter 4). LMFL supplementation demonstrated no effect on the relative abundance of microbiota in the solid or liquid associated populations. This suggests that microbiota do not utilise the LMFL as a substrate for microbial growth but instead the LMFL increases microbial activity/ metabolism. This perhaps explains why LMFL supplementation had an increasing effect on microbial fermentation within the rumen (section 7.3.3). However, as discussed in section 7.3.3 LMFLs

appear to have a short-term effect on rumen fermentation and is perhaps indicative of why no effect on microbial biomass was observed as a result of substrate being fermented rapidly. Furthermore, this may explain why positive effects are observed on animal performance in terms of live weight gain, as a result of increased molar concentrations of VFAs which are available for absorption by the host ruminant. Furthermore, this further highlights the use of the LMFLs in the long-term rather than in short-term in order to improve animal performance, as discussed in section 7.3.3.

The diet is known to have an influence on the structure and diversity of microbiota. Belanche *et al.* (2012) demonstrated cows fed diets at 80% of their nutritional requirement for protein to have lower, concentrations of bacteria, anaerobic fungi, and methanogens, a lower microbial diversity and reduced OM digestibility in comparison with cows fed diets nutritionally adequate in protein. Likewise, cows consuming diets high in fibre and low in starch had greater concentrations of protozoa, anaerobic fungi and methanogens and the microbial community was described as more complex in comparison with cows fed diets high in starch and low in fibre.

Previous research in cattle has demonstrated LMFLs to have an increasing effect on the digestibility of the diet (Greenwood *et al.*, 1998, Greenwood *et al.*, 2000, Löest *et al.*, 2001, Leupp *et al.*, 2005). It was hypothesised that LMFL supplementation would affect the structure, diversity and potential functionality of the solid associated bacterial population and in turn result in a population capable of increasing the degradation and fermentation potential of forage. This hypothesis was tested by identifying bacteria and their relative abundances within populations via 16s rRNA next generation sequencing. LMFL supplementation did not affect the structure, diversity or predicted functionality of the solid associated bacterial population associated with carbohydrate or protein metabolism. Likewise, pre-adapting the rumen to the LMFL had no effect on the structure, diversity or functionality of the solid associated bacterial population as predicted by CowPI (Wilkinson *et al.*, 2018). This agrees with observation made *in-vitro* and *in-sacco* in which LMFL supplementation had no effect on the degradation of forage in the rumen (section 7.3.1) or total tract digestibility of the diet (section 7.3.2). Likewise, supplementation had no effect on the activity of enzymes, amylase, xylanase and carboxymethyl-cellulase associated with the solid associated microbial population. This was surprising regarding LMFL supplementation enhancing rumen fermentation (section 7.3.3). However, microbiota of the liquid associated population are responsible for the fermentation of soluble and simple carbohydrates (Belanche *et al.*, 2017) such as those present within LMFLs. Therefore, any effect of LMFLs on the microbial community structure and diversity are likely to be associated with this community although these effects may only be short term. It must be remembered that the molecular work in this thesis was conducted in *in-vitro* experiments involving rumen microbiota being obtained from filtered rumen fluid. Therefore, the starting population for all experiments was initially from the

liquid fraction. As a result further molecular research should be conducted *in-vivo* so that microbiota samples can be obtained from the "true" solid and liquid fractions.

All in all, these results are indicative of LMFL supplementation having no effect on the structure, diversity or potential activity of the solid associated bacterial population in mature non-productive sheep. Likewise, supplementation has no effect on carbohydrate and protein metabolism of the solid associated population in mature non-productive sheep. As discussed in section 7.3 LMFL supplementation appears to effect metabolism (digestibility of forage) when the diet is nutritionally limiting. Therefore, further research is required to determine the effect of LMFL supplementation on the structure, diversity and functionality of the solid associated population when the diet is nutritionally limiting. This could be conducted in growing animals or animals with nutritional requirement for production (breeding). Furthermore, LMFLs have demonstrated to have a transient effect on rumen fermentation therefore further studies are required to investigate the effect of LMFL supplementation on rumen microbiota early after supplementary consumption.

7.5 Further Research

This thesis has demonstrated LMFL supplementation to have the potential to have positive effects on the performance and reproductive performance of breeding ewes in the uplands (Chapter 3). Further research is required to determine the utilisation of the LMFLs in conjunction with forage availability and quality. For example, commercial breeding ewes in the uplands (Chapter 3) demonstrated utilisation of the LMFLs to be greater when ewes were in the last trimester of pregnancy and when forage was limiting. In contrast, mature non-productive sheep exhibited signs of substitution of forage for the LMFL when the diet was sufficient for maintenance requirement (Chapter 6). Having such information around animal dietary choice would be beneficial to help determine when is best to feed the LMFL, the duration of time to feed the LMFL for and help with block specification for improving production efficiency.

LMFL supplementation demonstrated no effect on the appetite, metabolism or rumen microbiota of mature non-productive sheep which was attributed to the diet being nutritionally sufficient for maintenance requirement. Therefore, further studies are required to investigate the effect of LMFL supplementation on the appetite and metabolism of sheep when the diet is limiting in nutritional quality. This could be performed by using growing lambs or animals with a high nutritional requirement for production (breeding).

This thesis demonstrated LMFLs to be utilised in the rumen as a substrate for rumen fermentation, however this effect is only a short-term effect. Moreover, because the effect of LMFL was believed to

be targeted towards increased fibre degradation, this thesis focused on the characterisation of the solid associated population whereas the liquid associated population is associated with the fermentation of carbohydrates in solution. Therefore, further research is required to investigate the effect of LMFL supplementation on the liquid associated microbial population. Furthermore, microbiota from the kingdom bacteria were the focus of this thesis, therefore further research is required to determine the effect of LMFL supplementation on the structure and diversity of other microbial kingdoms such as methanogens, anaerobic fungi and protozoa.

From comparing studies within this thesis with those in the literature difficulties were faced in that the LMFLs used had different specifications and contained different dietary ingredients. Therefore, further research is required to understand which aspect of the LMFL has the greatest effect on animal metabolism and performance. For example, comparisons could be made between LMFLs with different dietary ingredients or inclusion rates of ingredients. Likewise, the manufacturing process of LMFLs is unique in that the raw ingredients are is dehydrated into a semi-solid material. Therefore, further research is required to determine the effect of the manufacturing process on metabolism.

7.6 Overall Conclusion

In conclusion, LMFLs are a good form of supplementation for forage-based diets providing a highly palatable form of additional energy, protein, vitamins and minerals to the diet with little labour requirement at feed out. LMFLs have the potential to improve voluntary forage intakes and digestibility of the diet when forage is nutritionally limiting and in turn have positive effects on the performance of animals (Figure 7.3). Thereby, highlighting the use of LMFLs as forage balancers. However, LMFL supplementation has no effect on the voluntary forage intake or digestibility of the diet when the diet alone is nutritionally sufficient for maintenance requirement. Therefore, supplementation has the potential to increase the live weight of animals most likely because of the additional nutrition the LMFL provides to the diet aiding fat deposition.

LMFLs act as a substrate for fermentation by rumen microbiota. However, pre-adapting the rumen to the LMFLs has no effect on rumen fermentation, suggesting microbiota to only be stimulated at the point of consumption. This highlights the use of LMFLs as a long-term supplement rather than a short-term supplement due to the LMFLs being utilised by microbiota rapidly post consumption. Furthermore, the use of LMFL supplementation as a long-term supplement has the potential to improve animal performance as a result of increased availability of fermentation products (VFAs) available for absorption by the host ruminant.

When the diet is nutritionally sufficient for requirement, LMFL supplementation has no effect on the growth of microbiota of the solid or liquid associated populations. LMFL supplementation has no effect on the structure, diversity or potential functionality of the solid associated bacterial population. However, LMFL supplementation has an enhancing effect on microbial metabolism at point of consumption in which rumen fermentation is enhanced. Therefore, it is anticipated that LMFL supplementation has a stimulating effect on microbiota of the liquid associated population albeit short-term.

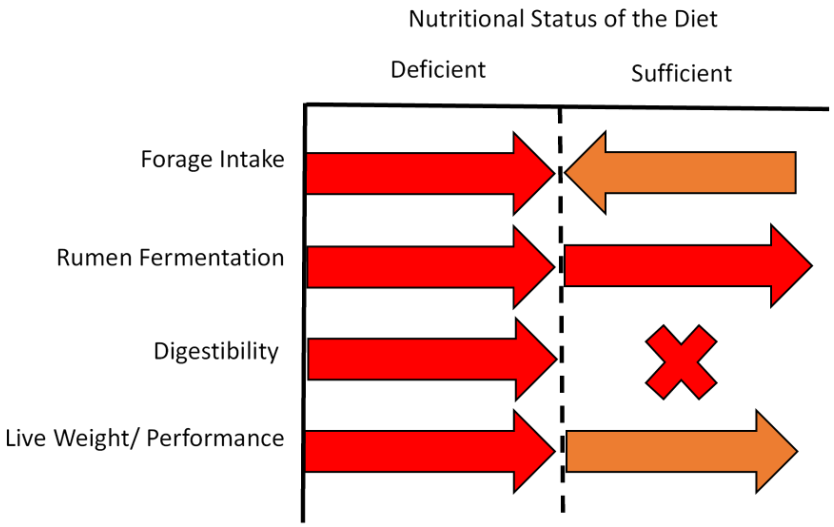


Figure 7.3: Schematic diagram identifying the main effects of low moisture feed lick supplementation on metabolism when the diet is nutritionally deficient or sufficient for production and maintenance requirements. Arrows indicate the direction of the effect and x indicates no effect, arrows in orange indicate a tendency for an effect and arrows in red indicate a significant effect.

7.7 Key Findings for the Farming Community

From the observations made in this thesis and in line with the literature, the supplementation of sheep with low moisture feed licks (LMFLs) has demonstrated the potential to have positive effects on animal performance in which;

- LMFL supplementation has the potential to increase the digestibility of forage and in turn voluntary forage intakes when forage is nutritionally limiting for production requirement, thereby acting as a forage balancer

- Supplementation with LMFLs at grazing demonstrated;
    - The effect of helping to maintain and improve animal live weight
    - A tendency to increase scanning percentage of which a larger proportion of twin bearing ewes were observed
    - Supplemented ewes to have a shorter lambing period in comparison to un-supplemented ewes, however this was just an observation

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